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Transcriptional Profiling of Non-injured Nociceptors after Spinal Cord Injury Reveals Diverse Molecular Changes

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ABSTRACT

The work presented here begins to provide insight into the underlying mechanisms that contribute to the transition from acute to chronic pain. Our work employed spinal cord injury (SCI) as a unique model to study chronic pain, and to enhance our knowledge of the transcriptional changes that occur following injury. The studies herein support the idea that SCI alters genetic, cellular, and molecular pathways at distal sites (below the site of injury) such as skin, muscle, and the sensory neurons that project to these regions. We provide evidence that a specific population of sensory neurons located in distal dorsal root ganglia exhibit significant transcriptional changes that are relevant to the onset of chronic pain. By enhancing our knowledge regarding the changes that occur at different time points, within different tissues, and within specific cell populations, we can better understand why chronic pain develops weeks to months after injury in patients. In particular, our work on transcriptional profiling of specific sensory neurons projecting to the skin below the level of injury, in comparison to whole tissue analysis, emphasizes how injury does not impact all cell populations in the same way. A multifaceted approach is essential for the development of new treatment strategies and models for patients, in an effort to prevent irreversible changes in pain signaling before they occur.

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Transcriptional Profiling of Non-injured Nociceptors after Spinal Cord Injury Reveals Diverse Molecular Changes

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CHAPTER 1

Chronic pain following spinal cord injury: Current approaches to cellular and molecular mechanisms

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ABSTRACT

Traumatic spinal cord injury (SCI) has devastating implications for patients, including a high prevalence of chronic pain. Despite advancements in our understanding of the mechanisms involved post-SCI, there are no effective treatments for chronic pain following injury. The development of new treatment interventions for pain is needed, but this requires improved models to assess injury-related cellular, neurophysiological and molecular changes in the spinal cord. Here, we will discuss recent animal models for SCI, molecular screening for altered patterns of gene expression, and the importance of injury severity and timing after SCI.

Introduction

Traumatic spinal cord injury (SCI) has overwhelming implications for patients and caretakers. There are currently over 1 million people affected by SCI in North America, with lifetime costs per patient reaching up to $4.6 million (Ahuja et al., 2017; Siddall et al., 1999; Warner et al., 2018). Most treatment options emphasize rehabilitation and neuroprotection; however, an average of 65% of patients report chronic pain, with an estimated 33% describing their pain as severe or excruciating (Siddall et al., 1999). Animal models have been utilized to study a myriad of therapeutic interventions, with a primary focus on improving neurological outcomes after injury. However, a significant concern in promoting axonal repair within the central nervous system (CNS) is the prospect of the development of neuropathic pain. Neuropathic pain is pain caused by a lesion or disease of the nervous system. It has several distinguishing features, including lesions of nervous tissue, pain in an area of sensory loss, pain in response to a normally non-noxious stimulus (allodynia), increased pain in response to noxious stimuli (hyperalgesia), and unpleasant or abnormal sense of touch (dysesthesia) (Finnerup et al., 2001; Warner et al., 2018). Despite an improved understanding of the mechanisms involved in the pathophysiology observed following SCI, there are still no effective treatments for chronic pain (Hachem et al., 2017). This review will discuss the unique features of pain that can accompany SCI as well as current animal models and approaches that are being employed to better develop treatment methods for SCI patients.

Gross anatomy of the pain pathway

Sensory fibers that innervate specific regions of the body arise from cell bodies within the trigeminal ganglion and dorsal root ganglia (DRG). Neurons within the DRG (collections of sensory neurons just outside the spinal cord) do not have dendrites, but have a single axon that bifurcates, with one branch projecting to the periphery and the other projecting to the CNS. The peripheral branch is functionally a dendrite, carrying information toward the cell body, while also

having “axonal” properties in conducting action potentials. These neurons are therefore considered “pseudo-unipolar” (Le Pichon and Chesler, 2014).

Conventionally, neurons within the DRG are distinguished by cell body size, degree of myelination, and terminal location within the dorsal horn of the spinal cord (Kandel, 2012). Using these principles, somatosensory neurons have been classified into four fiber types, Aβ, Aδ, C-fibers, and proprioceptors (Fig. 1-1A). Each class has specialized roles in sensation. DRG cell bodies with the largest diameter (>50μm) represent the myelinated, rapidly conducting (30-70 m/s) Aβ fibers that respond to innocuous stimuli, such as light touch. These fibers do not respond to noxious stimuli. Aδ fibers have medium-diameter cell bodies, are lightly myelinated and are thought to conduct “first” pain, specifically the rapid (5-30 m/s), sharp pain that occurs following noxious stimuli. C-fibers have the smallest cell bodies (10-30 μm), are unmyelinated, slow conducting (0.5-2 m/s) and convey “second” or delayed pain after noxious stimuli (Fig. 1-1B). Most C-fibers are polymodal, and respond to thermal, mechanical, or chemical stimuli (Julius and Basbaum, 2001; Le Pichon and Chesler, 2014; Purves, 2017). C-fibers are the most abundant neuronal class within the DRG, and make up more than half of all somatosensory neurons (Purves, 2012). Previous studies have validated that C-fibers, or nociceptors, respond to specific stimuli such as heat or chemicals, but not to non-noxious stimuli, such as light touch (Burgess and Perl, 1967). C-fibers project to interneurons within the dorsal horn of the spinal cord that project to the somatosensory cortex *via* the thalamus, transmitting information about painful stimuli (Basbaum et al., 2009) (Fig. 1-1C).

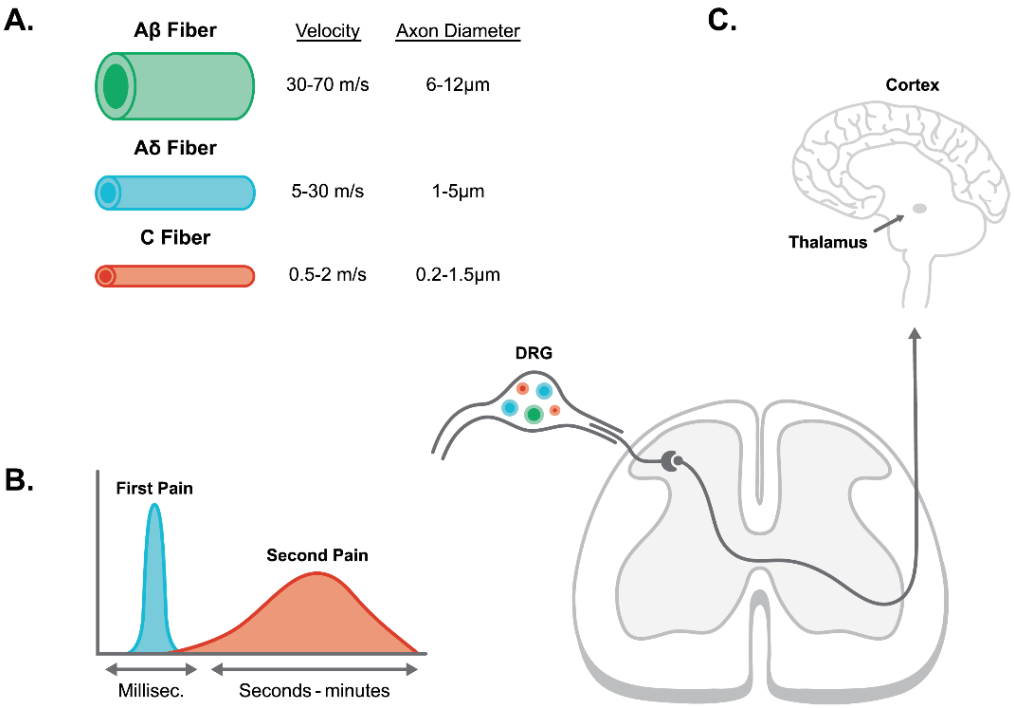

Figure 1-1. (A) Somatosensory neurons can be divided by cell body size and degree of myelination. These include large-diameter Aβ myelinated fibers, medium diameter Aδ fibers, and small diameter unmyelinated C-fibers. **(B)** Conduction velocity is related to degree of myelination. Following noxious stimuli, Aδ fibers account for the immediate "fast" pain that occurs within milliseconds, and C-fibers are responsible for secondary "slow" pain in response to noxious stimuli. In neuropathic pain, secondary pain persists, even in the absence of noxious stimuli. **(C)** The dorsal root ganglia (DRG) are comprised of a heterogeneous population of sensory neuron cell bodies that project to both the periphery and the spinal cord. Efferent projections synapse onto second order neurons within the dorsal horn of the spinal cord and project to the thalamus and somatosensory cortex for the perception of pain.

Cellular anatomy of the pain pathway

DRG neurons have been characterized both by gene expression and protein expression as well as cellular function. Studies have utilized immunofluorescence to broadly distinguish A-fibers using antibodies specific to neurofilament 200 proteins and the antibody peripherin to identify unmyelinated C-fiber subpopulations (Aletta et al., 1988; da Silva Serra et al., 2016). C-fibers are further classified into two general categories, peptidergic and non-peptidergic (Barabas et al., 2014). The peptidergic class is demarcated by the expression of neuropeptides such as calcitonin gene related-peptide (CGRP) or substance P. The non-peptidergic class is distinguished by the binding of isolectin B4 (IB4) to α-D-galactose carbohydrate residues on the cell membrane (Averill et al., 1995). Following injury, altered gene expression and protein expression may cause overlap between these groups (Neumann et al., 1996; Ueda, 2006).

C-fibers terminate as free nerve endings on peripheral targets in the skin, organs, and bone. Centrally, they project to the superficial laminae (I, II) of the dorsal horn of the spinal cord and are responsible for the initial stages of pain processing (Basbaum et al., 2009). Previous work has utilized wheat germ agglutinin-horse radish peroxidase conjugate (WGA-HRP) to label this smaller population of cells within the DRG as well as their afferent projections into the superficial laminae of the dorsal horn (LaMotte et al., 1991).

Whereas most neurons typically have biochemically distinct dendrites and axons, the unique structure of the DRG allows for uniform protein distribution, since both the central and peripheral terminals send and receive messages. The central terminal projections are dependent on calcium for neurotransmitter release, and the peripheral terminal delivers molecules such as CGRP and substance P to the local tissue (Basbaum, 2000). Each afferent fiber type projects to anatomically distinct laminae; Aβ afferents project to lamina III, IV, and V, Aδ fibers project to lamina I and lamina V, and C-fibers project to the superficial laminae I and II (Fig. 1-2A). The dorsal horn is

further organized by C-fiber subtype projections, where the peptidergic class of neurons terminates within lamina I and the outer region of lamina II, and the non-peptidergic afferents terminate in the inner layer of lamina II. These laminae are further organized by electrophysiological characteristics, where the ventral portion of lamina II is composed largely of excitatory interneurons that express protein kinase C (PKC) γ (Fig. 1-2B) (Basbaum et al., 2009).

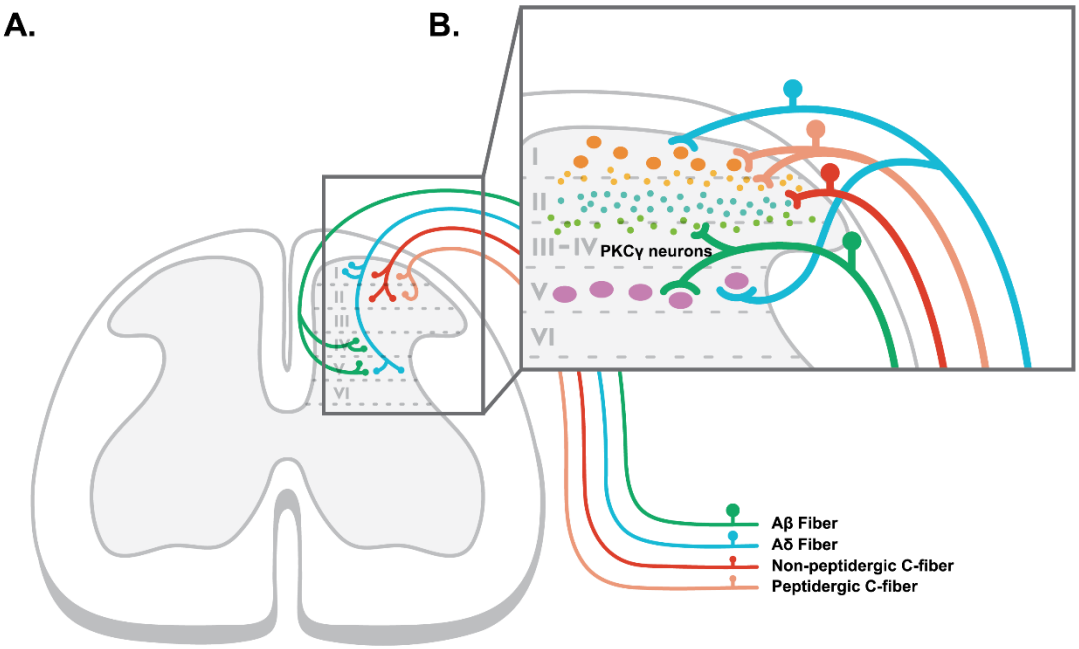

Fig. 1-2. (A) Different subtypes of DRG neurons terminate within discrete laminae of the dorsal horn of the spinal cord. **(B)** Fast Aβ fibers (green) project onto PKCγ interneurons located within inner laminae II and also project more deeply to lamina V. Slower Aδ fibers (blue) terminate in lamina I and V, while the slowest C fibers (red and orange) project more superficially. Non-peptidergic C-fibers (red) project to interneurons within inner lamina II, and peptidergic C-fibers (orange) terminate onto interneurons in lamina I and the outer lamina II. Figure adapted from (Basbaum et al., 2009).

Nociceptors

Nociceptors are heterogeneous in both their physiology and cellular properties. The sensory endings of their primary afferents project to various peripheral tissues, including skin, muscle, joints, and viscera. During development, immature sensory neurons evolve via dedicated gene programs that orchestrate neuronal subtype specific characteristics. These neurons eventually mature into a diverse population of sensory neurons that respond to a variety of chemical, mechanical, and thermal environmental cues (Lallemend and Ernfors, 2012). Each subgroup exhibits stereotypical patterns that terminate within the dorsal horn of the spinal cord; in addition, they terminate in specialized end structures or as free nerve endings in the periphery.

DRG sensory neuron subtypes can be distinguished by their expression of neurotrophic factor receptors: tropomyosin-receptor-kinase A (TrkA), TrkB, TrkC, and Ret (Ret proto-oncogene) bind nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT3 [gene name NTF3]) and glial-derived neurotrophic factor (GDNF) family ligands, respectively. These receptors are necessary for appropriate peripheral innervation of tissue targets, cell survival, and the expression of ion channels and receptors. Most neurons with small-diameter, unmyelinated axons develop following the expression of neurogenin 1 (Ngn1) during neurogenesis at embryonic days 9-10 (Lallemend and Ernfors, 2012). An increase in TrkA expression prompts the early stages of differentiation, while an increase in runt related transcription factor 1 (Runx1) and either the proto-oncogenes Met, Ret, or protein coding gene transient receptor potential cation channel 8 (Trpm8) further specifies subtypes and central innervation. Additional specification and subclass refinement occurs postnatally (Lallemend and Ernfors, 2012). The peptidergic sensory neurons express TrkA and respond to NGF while the non-peptidergic sensory neurons express ATP-gated P2X3 purinergic receptors (Julius and Basbaum, 2001).

A wide range of stimuli can activate sensory neurons. Previous studies have used retrograde tracing, immunohistochemistry, and electrophysiology to identify the various properties of neurons innervating distinct tissues (da Silva Serra et al., 2016). However, efforts to attribute pain modalities and behavior to individual sensory neurons have proven to be a difficult task. In conjunction with the involvement of multiple systems following injury, the diversity of this population has made it challenging to understand cellular pain mechanisms (Abrahamsen et al., 2008).

Structural changes following SCI

While a consequence of all types of pain is modification of the pain circuit, it is important to distinguish differences between the outcomes of short-term pain, such as inflammatory pain, vs. chronic pain, including neuropathic pain. Following tissue injury, primary sensory neurons exhibit alterations in excitability, largely mediated by the activation of intracellular signaling pathways *via* phosphorylation of receptors or ion channels. These changes cause posttranslational modifications that can alter cell-surface expression of channels within the DRG or dorsal horn of the spinal cord (Woolf and Salter, 2000). This form of plasticity is modulatory, and is reversible. Although neuroplasticity is essential in spontaneous recovery following SCI, these compensatory shifts may also produce negative consequences, such as neuropathic pain (Finnerup, 2013). Long-term changes within the pain circuit appear during neuropathic pain due to plasticity that causes permanent modifications of the pain pathway. This latter type of plasticity is likely representative of what occurs in SCI patients experiencing chronic pain. These long-lasting shifts are supported by changes in the expression of neurotransmitters, receptors, and ion channels, but also changes due to neuronal survival and subsequently system connectivity (Woolf and Salter, 2000). The result of these modifications is a system that no longer has normal stimulus-response characteristics.

Although precise mechanisms responsible for driving chronic pain are not well understood, it is likely that multiple processes are involved. These include functional as well as structural neuroplasticity within the CNS that culminates in increased neuronal excitability (Siddall and Loeser, 2001). Variations in neurotrophic factor expression and neuronal damage from an increase of excitatory amino acid levels both play a role in modulating changes after injury (Bakhit et al., 1991; Christensen and Hulsebosch, 1997b). Immunohistochemistry studies have shown that SCI can also physically alter primary sensory neurons; nociceptors immunoreactive for CGRP exhibit sprouting of new branches within the dorsal horn, which also contributes to the development of new abnormal connections (Hou et al., 2009; Polistina et al., 1990). It is important to note that sprouting has not been found in all models of SCI (Kalous et al., 2007, 2009).

It is well established that SCI produces increased extracellular concentrations of glutamate, as well as inflammatory cytokines and reactive oxygen species (ROS). SCI not only elicits changes within the neuronal population, it also causes extensive alterations in spinal microglia and astroglia; these changes may promote pain-related behaviors as well (Carlton et al., 2009; Gwak and Hulsebosch, 2011; Hains and Waxman, 2006; Walters, 2012). Microglia are present after injury, as evidenced by the increased levels of proinflammatory cytokines that are detectable within the first several minutes of injury. These changes promote an increase in extracellular glutamate to excitotoxic levels within a similar timeframe (Rowland et al., 2008). During the acute phase of injury, astrocytes surround the region of injury and proliferate in an attempt to prevent further damage. However, over time the increased presence of astrocytes becomes detrimental, creating a glial scar and preventing regeneration (Hachem et al., 2017).

Clinical presentation of SCI pain

SCI can be categorized into two phases, primary and secondary injury (McDonald and Sadowsky, 2002; Rowland et al., 2008; Tator, 1995). The immediate results following injury are due to direct physical trauma to the spinal cord, and result in spinal shock and complete loss of motor and

sensory function below the level of injury. This physiological response is accompanied by loss of tendon reflexes and absence of the sphincter reflex (Hachem et al., 2017). A cascade of secondary events follows, expanding the region of neural injury and worsening neurological outcomes (Norenberg et al., 2004; Yip and Malaspina, 2012). This secondary event, or injury, is used to describe the delayed and progressive multitude of physiologic, biochemical, and intracellular changes that occur following the primary spinal cord injury (Tator and Fehlings, 1991).

During the early stages of the secondary response, inflammatory cells (macrophages, microglia, T-cells, neutrophils) enter the site of injury. Studies conducted in rodents have shown that within 6 hours this response triggers the release of cytokines such as interleukin-1α (IL-1α), IL-1β, IL-6, and tumor necrosis factor-α (TNF-α), which can remain elevated for up to 4 days (Nakamura et al., 2003; Ulndreaj et al., 2016). Phagocytic inflammatory cells (largely macrophages and neutrophils) also release reactive oxygen species, causing oxidative DNA damage and ATP release, ultimately contributing to delayed apoptosis, cord edema and a pro-inflammatory state (Ahuja and Fehlings, 2016; Hausmann, 2003; Waxman, 1989). This disruption of the blood-spinal cord barrier permits the disequilibrium of ionic homeostasis of the cord to go unchecked, and activates calcium-dependent proteases, mitochondrial dysfunction, and finally apoptosis (Schanne et al., 1979).

Oligodendrocytes are among the many cell types susceptible to cell death, and loss of these CNS myelinating cells has been observed both at the site of injury as well as distant to the lesion epicenter (Beattie et al., 2000; Crowe et al., 1997). Excitatory amino acids such as glutamate and aspartate are also released as a consequence of cell death, and further propagate excitotoxicity (toxicity to neurons caused by excess calcium entry *via* excitatory neurotransmitters) and glial and neuronal death within the region surrounding the injury (Ahuja et al., 2017; Li and Stys, 2000; Liu et al., 2015).

Pathophysiological processes activated by the primary injury contribute to the more protracted secondary injury phase (Rowland et al., 2008). It is useful to examine secondary injury during different time periods following the primary injury. The immediate 0-2 hours after injury are characterized by swelling of the spinal cord and cell death. From 2-48 hours post injury (acute window), hemorrhaging of the cord, edema, and inflammation occur. From 2 days- 2 weeks post injury (sub-acute window) as well as during a transitional period (2 weeks- 6 months after injury), scarring from astrocytes and axonal sprouting occur. Finally, a chronic secondary injury continuum occurs, with the formation of scars, Wallerian degeneration (nerve process death extending from an injury to the distal process), and injured axons (Rowland et al., 2008). This prolonged secondary injury cascade produces a harsh post injury environment and results in the unique pathophysiology of SCI that obstructs regeneration and healing and promotes the development of chronic pain (Ahuja et al., 2017).

When patients are asked about complications associated with SCI, pain is rated as the third most important symptom, ranking just behind decreased ability to walk or move and decreased sexual function (Siddall et al., 2003). The current treatment options for chronic pain consist of systemic steroid therapy, such as methylprednisolone, early surgical decompression, and early mobilization for rehabilitation (Bracken et al., 1984; Bracken et al., 1990; Fehlings et al., 2012; Lam et al., 2007). Because pain is severe, chronic, and resistant to treatment, animal studies are necessary in order to develop strategies for better pain management, or preferably pretreatment of chronic pain. However, there are no predictive measures for chronic pain or effective treatments (Zeilig et al., 2012). It is likely that pain develops within weeks to months after injury, or perhaps even earlier, and that many patients are being treated after the development of pain has already begun (Zeilig et al., 2012). Due to the bi-phasic nature of the injury response following SCI, contiguous delivery interventions during the early post-injury stage would probably have a positive

impact on long-term recovery, both functionally but also as a preventative approach to the development of chronic pain.

Types of pain following spinal cord injury

Pain elicited by SCI is difficult to manage and is a priority for patient treatment (Gaudet et al., 2017; Nepomuceno et al., 1979). Patients with chronic pain following SCI most commonly report pain in segments near the site of injury (at-level pain) and below the level of injury (below-level pain) (Finnerup and Jensen, 2004; Siddall et al., 2003). Pain above the level of injury does occur, but is typically due to upper extremity pain from overuse of muscles, rather than a result of the injury itself (Dalyan et al., 1999; Hagen and Rekand, 2015; McCasland et al., 2006). The most predominant trait reported for at- and below-level pain is burning pain; however, there are at least five types of pain that may arise after injury, and these are important to consider as they may contribute to long term pain phenotypes (Widerstrom-Noga et al., 2001).

Acute pain

Acute pain is defined as a cascade of events aimed to fight infection, to prevent further damage, and to initiate repair. This occurs during the primary phase of SCI and involves inflammatory responses, neuronal changes, as well as peripheral and nerve sensitization. These alterations enhance nociceptive responses in an effort to limit further injury to the lesion. Under normal conditions, acute pain functions to prevent further damage and wanes as healing progresses (Voscopoulos and Lema, 2010).

Inflammatory pain

Inflammatory pain can be a form of acute pain, which is triggered in response to tissue damage and inflammation, and is characterized by hypersensitivity at the site of injury and nearby tissue as a result of increased excitation of nociceptors (Linley et al., 2010; Woolf and Salter, 2000).

Although inflammatory pain occurs during the primary or acute phase of injury, and may also be a contributing factor to chronic pain, it typically dissipates as the disease process heals. After SCI, cell bodies of nociceptors (sensory neurons responding to painful stimuli) are exposed to the macrophages and T-cells that have infiltrated one or more DRG near the site of the spinal lesion (McKay and McLachlan, 2004). DRGs have a much higher vascular permeability than the blood-brain or blood-nerve barriers (Abram et al., 2006; Jimenez-Andrade et al., 2008), leaving neuronal cell bodies and satellite glial cells within the DRG exposed to both blood and cerebrospinal fluid and the inflammatory factors that are released after SCI (Blum et al., 2014; Cheng et al., 2014; Huang et al., 2013). Many studies have evaluated the therapeutic efficacy of inhibiting various inflammatory factors to reduce tissue injury and pain associated with spinal cord trauma. One study used TNF-α knockout mice in a vascular clip model of SCI to demonstrate its role in the development of inflammation and the pathogenesis of SCI (Genovese et al., 2008). Additional studies have shown that specific channels, such as sodium channels $Na_v1.8$ and $Na_v1.9$ (encoded by genes SCN10A and SCN11A) are critical for inflammatory pain, but not neuropathic pain (Abrahamsen et al., 2008; Cook et al., 2018). While certain groups have demonstrated mechanisms that are exclusive to inflammatory pain, others have shown an overlap between inflammatory and chronic pain. Lalisse *et al*. used ATP-gated purinergic receptor P2X4-deficient mice to determine that the receptor is expressed within DRG neurons and to establish a role for P2X4. The study demonstrated that during continued inflammation, P2X4 mediates the release of neuronal BDNF, which contributes to hyperexcitability during chronic inflammatory pain (Lalisse et al., 2018).

Chronic pain

Chronic pain is characterized by persistent activation of nociceptors in the periphery, which produces peripheral and eventually central sensitization (Voscopoulos and Lema, 2010). Chronic pain may also result from abnormal firing of myelinated sensory neurons that are not normally

responsible for conveying noxious stimuli, that have defective sodium channels following injury, or from overly active central circuits, possibly *via* wide dynamic range (WDR) neurons in the spinal cord (Basbaum et al., 2009). Although the transition to chronic pain is widely thought to occur after the onset of acute pain, it is possible that both acute and chronic pain mechanisms emerge simultaneously (Price et al., 2018). The shift to chronic pain is not well understood, in part because many different systems contribute to the development of similar phenotypes that present as allodynia and hyperalgesia. Persistent pain can present as both hypersensitivity (allodynia and hyperalgesia) and spontaneous pain. Hypersensitivity results from a decrease in the threshold for nociceptors to fire action potentials in response to normally non-noxious stimuli or to produce an amplified response to noxious stimuli. This is well documented particularly *via* activation of transient receptor potential (TRP) channels after inflammation (Linley et al., 2010). These secondary mechanisms not only include changes in the excitability of primary sensory neurons *via* post-translational modifications of receptors and ion channels, but also alterations in expression, connectivity, and neuronal survival, all of which modify normal stimulus-response characteristics of pain (Woolf and Salter, 2000). It is unclear whether treatment plans should be aimed at preventatively targeting mechanisms that underlie the transition to chronic pain, or if there is a way to resolve and reverse chronic pain after it has already developed.

Nociceptive pain

Nociceptive pain is the most prevalent type of pain after SCI and occurs during both the acute and chronic phase (Finnerup, 2013). Nociceptive pain is produced by activation of peripheral nociceptors stimulated by continuous tissue damage, and not from persistent painful insults. Analgesics, such as non-steroidal anti-inflammatory drugs (NSAIDs) and physical therapy are effective in treating nociceptive pain, but not neuropathic pain (Eide, 1998).

Neuropathic pain

Neuropathic pain is elicited by lesions or diseases of the nervous system that alter its function (Maynard et al., 1997). This syndrome is characterized by spontaneous and evoked pain (Baron, 2006). Like inflammatory pain, neuropathic pain is described, in part, by hypersensitivity at the site of injury as well as in nearby uninjured tissue. Because this type of pain is triggered by alterations in the function of the somatosensory nervous system, pain is amplified and appears spontaneously (Ducreux et al., 2006). Neuropathic pain frequently results from SCI and develops in over half of SCI patients, typically within the first year after injury, and has a tendency to progress into chronic pain (Finnerup, 2013). Neuropathic pain is divided into at-level pain, and below-level pain (Finnerup, 2017; Shiao and Lee-Kubli, 2018). At-level pain is demarcated by its existence within a region of one dermatome rostral (up the spinal cord) and three dermatomes caudal (down the spinal cord) to the site of injury. Below-level pain is defined as the presence of pain emanating from three dermatomes below the level of injury. Patients most commonly describe below-level pain as burning, tingling, or pins-and-needles sensation in the absence of sensory stimuli (Finnerup et al., 2016).

There are several mechanisms thought to contribute to neuropathic pain including the development of maladaptive plasticity in the nervous system, aberrant sensory neuron activation, increased synaptic transmission, alterations in synaptic connectivity, as well as neuro-immune interactions (Baron, 2006; Eide, 1998). Additional factors, such as genetic variations, gender, and age can all impact the development of persistent pain (Costigan et al., 2009; Walters, 2018). A defining feature of neuropathic pain is that it remains even after the initial injury has healed, becoming an uncontrolled, inappropriate action of the nervous system rather than an appropriate response to the condition (Woolf and Salter, 2000). A better understanding of the mechanisms responsible for abnormal recovery can offer precise therapeutic opportunities to individuals with neuropathic pain.

Nociceptor mechanisms in response to SCI

Central alterations

The somatosensory system is structured in such a way that specialized primary sensory neurons that encode low intensity stimuli only activate pathways in the CNS that convey proprioception, and sensory neurons that encode high intensity stimuli only activate pathways that lead to pain perception (Woolf et al., 2004). However, following central alterations, sensitization occurs and noxious stimuli are no longer necessary to produce a nociceptive response (Woolf, 2011). Previous studies support this idea of the development of central sensitization of dorsal horn neurons after SCI (Christensen and Hulsebosch, 1997a). If this does occur, this provides an explanation for the development of mechanical and thermal hypersensitivity following injury.

There are several proposed mechanisms for the cause of central sensitization. These include enhanced or prolonged discharge of C-fibers, spontaneous activity from DRG sensory neurons, disinhibition *via* loss of gamma-aminobutyric acid (GABA) and glycine within the spinal cord, increased efficacy of previously weak synapses, and a change in the presence of excitatory amino acids, peptides or other receptors (Basbaum and Wall, 1976; Bedi et al., 2010; Devor and Wall, 1981; Sluka and Westlund, 1993; Willis, 1993a; Willis, 1993b). All of these changes might occur over a period of several days or longer (Christensen and Hulsebosch, 1997a). After injury, central sensitization emerges, as the somatosensory system pathways converge and distort or amplify pain signals (Costigan et al., 2009). This central amplification takes place following reduced inhibition within the central system, and enhances the sensory neuron response in amplitude, duration, and spatial extent, as well as increasing synaptic efficacy, so that low threshold sensory inputs now activate the pain circuit (Costigan et al., 2009; Woolf, 2011).

Other research suggests that central sensitization can begin within seconds after injury by an increase of activity in nociceptors (Woolf, 2018). However, it is likely that prolonged inputs to

nociceptors over a period of days to months will produce a more persistent phenotype of hyperexcitability within the central nervous system. These sustained changes may be a greater contributor to the development of central sensitization, because inflammatory factors can activate nociceptors within the DRG, and can also produce signaling molecules, such as NGF, that have downstream effects on sensory neuron behavior (Leslie et al., 1995) (Fig. 1-3A). It is evident that the process of central sensitization is complex and reflects the involvement of numerous mechanisms at multiple sites within the spinal cord, brainstem, and cortex.

The increase of excitatory neurotransmitters, glutamate in particular, is also a contributing factor in the development of central sensitization, and is associated with allodynia and hyperalgesia in patients that report chronic pain (Latremoliere and Woolf, 2009) (Fig. 1-3B). One study demonstrated the involvement of type 4 metabotropic glutamate receptors (mGluR4 [GRM4]) and thus the ability to regulate glutamatergic signaling in the spinal cord. This was done by inhibiting glutamatergic transmission in both C-fibers and afferent terminals in the inner lamina II of the dorsal horn *via* coupling of Cav2.2 channels (Latremoliere and Woolf, 2009; Vilar et al., 2013). It is possible that the sensory neurons that contribute to the C-fiber population include a variety of chemical and structural differences in their plasma membranes that might affect cell signaling.

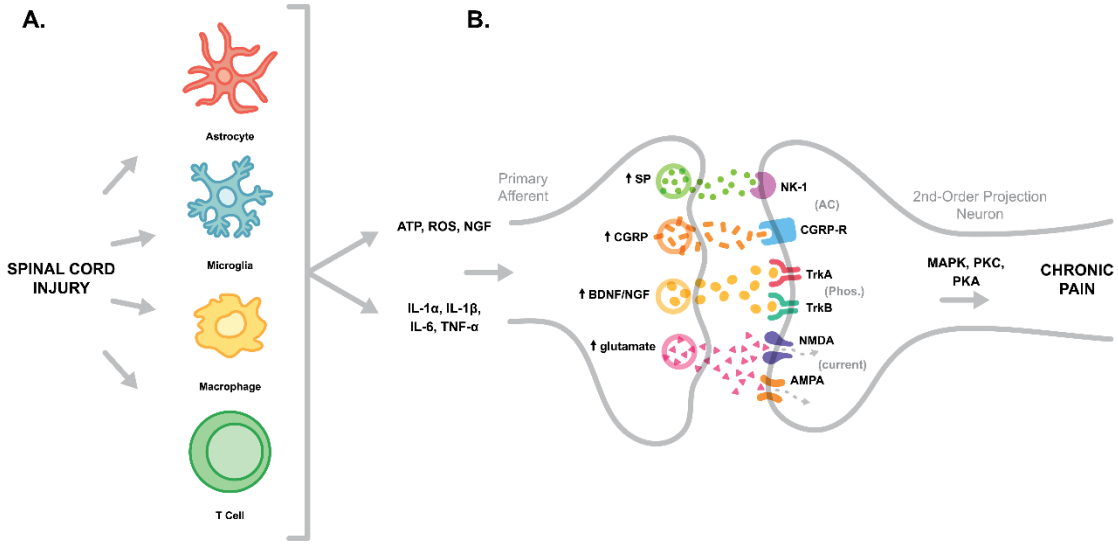

Figure 1-3(A) Depiction of the contribution of neuro-immune interactions in the development of pain. Following SCI, mediators such as IL-1α, IL-1β, IL-6, TNF-α, NGF, ATP, and reactive oxygen species (ROS) are released from invading astrocytes, microglia, macrophage, and T-cell populations. Released factors act directly on terminals of primary afferents that express receptors for these mediators. **(B)** After injury, C-fibers and some Aδ-fibers release a variety of neurotransmitters including substance P (SP), calcitonin-gene related peptide (CGRP), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and excess glutamate onto secondary projection neurons located within the superficial dorsal horn. As a result, NMDA and AMPA receptors in the postsynaptic neuron allow an increase in current (Ca^{++} and Na^{+}) to enter the cell. Activation of postsynaptic TrkA and TrkB receptors increases phosphorylation (Phos.) of downstream targets, and activation of CGRP receptors (CGRP-R) and SP receptors (NK-1) increases adenylyl cyclase (AC) activity. This cascade of events activates calcium dependent signaling pathways and second messenger systems including mitogen-activated protein kinase (MAPK), protein kinase C (PKC), and protein Kinase A (PKA), all of which increases neuronal excitability, neuropeptide release, and gene regulation that facilitates the transmission of pain messaging to the somatosensory cortex. Loss of GABAergic and glycinergic inhibition further perpetuates hyperexcitability within the spinal cord (not shown here). Figure adapted from (Mantyh et al., 2011).

Spontaneous activity

Despite the presence of spontaneous pain as a common problem after injury, it is poorly understood. It has been proposed that prolonged depolarization of resting membrane potential, lowered threshold for action potential propagation, and spontaneous activity of nociceptor sensory terminals could contribute to spontaneous pain (Huang et al., 2013; Linley et al., 2010). Glutamate is the primary excitatory neurotransmitter in all nociceptors (Wozniak et al., 2012). Regardless of structural changes, the accumulation of excitatory neurotransmitters following SCI, in combination with the loss of normal inhibitory processes such as GABA and glycine, can result in functional changes such as spontaneous activity and increased evoked neuronal activity (Siddall and Loeser, 2001). Spinal injuries are more likely to elicit multiple central alterations, rather than just one, which can then impact primary afferents and trigger chronic pain. In addition to the increase in excitatory amino acids following tissue damage, SCI also severs descending inhibitory pathways, causing disinhibition and further promoting excitatory transmission within the spinal cord (Bruce et al., 2002; You et al., 2008). Loss of inhibitory interneurons reduces the amount of GABA and glycine in the spinal cord, alters the Cl^- potential in neurons receiving inhibitory input, and allows persistent excitability of central neurons within pain pathways (Gwak et al., 2008; Lu et al., 2008; Peirs and Seal, 2016). An increase in excitability is evident based on the upregulation of Na^+ channels in spinal dorsal horn neurons (Hains et al., 2003). It is not surprising that the increase in voltage-gated sodium channels that occurs after injury also contributes to the generation of ectopic activity in the nociceptor population, as evidenced by the robust effects of nonselective sodium channel blockers (Meier et al., 2003; Sheets et al., 2008). Additional studies have highlighted the importance of the voltage gated sodium channel, $Na_v1.7$. Patients with erythromelalgia (burning pain) have a mutation in the *SCN9A* gene, which encodes $Na_v1.7$, and display increased firing in their sensory neurons (Dib-Hajj et al., 2005). Other findings have implicated excess release of glutamate, substance P, and neurotrophic factors such as BDNF or NT-3 in central sensitization after injury (Tan and Waxman, 2012).

Work done by Bedi *et al*. used a SCI contusion injury model at T10 (thoracic segment 10) to demonstrate that spontaneous activity occurs in the terminals of nociceptors distal to the site of injury as early as 3 days after injury, continuing for up to 8 months (Bedi et al., 2010). This suggests that persistent increased excitability above and below the level of injury in nociceptors may support the development or maintenance of chronic pain after SCI (Walters, 2012). Ectopic discharges of primary afferent fibers projecting to the spinal cord may subsequently result in amplified responses, or sensitization of spinal neurons (Liu et al., 2001; Sukhotinsky et al., 2004).

Several studies have suggested that an increase of neuronal activity within the dorsal horn is the cause of pain after SCI. However, the underlying mechanisms are still unclear. Many agree that increases in glutamate following injury, as well as the upregulation of NMDA receptors, are important contributors to increased excitability after injury (Eide, 1998). A role for long-term potentiation (LTP) in the spinal cord dorsal horn after injury has also been proposed as a contributor to pain after SCI, since the increase in excitability of primary sensory neurons activated by tissue injury prompts central sensitization that is analogous to the process of LTP (Woolf, 2018). This model would equate chronic pain after SCI with the LTP that transpires during memory formation. Importantly, both processes involve NMDA receptors.

Studies have shown that inflammation or injury stimulates hypersensitivity, reduces nociceptive thresholds, and induces synaptic potentiation between C-fiber afferents and WDR second-order sensory neurons within the spinal cord. WDRs receive synaptic input for nociceptor afferent terminals as well as from GABA-releasing neurons and descending inhibitory projections (Baron, 2006). The loss of GABA after injury, in combination with an increase in AMPA and NMDA receptors after injury, can also trigger an increase in intracellular postsynaptic calcium concentration (Tan and Waxman, 2012). Increased intracellular calcium levels are known to

activate protein kinases involved in the induction of LTP (Tan and Waxman, 2012).

Several other mechanisms for spontaneous activity have been proposed, including low-threshold large myelinated sensory neurons, which generate spontaneous pain due to altered connectivity within the spinal cord. In addition, inflammatory signaling between the spinal cord, DRG, and blood is involved in producing hyperexcitability (Walters, 2018; Woolf et al., 1992). Satellite glial cells (SGCs) may also play a role in the development or maintenance of chronic pain (Huang et al., 2013). SGCs are the primary type of glial cells in sensory ganglia and form a sheath that surrounds neuronal cell bodies (Pannese et al., 2003). This cell type also expresses several ion channels, receptors, and adhesion molecules (Pannese, 2010). Sensory neurons within the DRG do not form synaptic contacts between each other; however SGCs surrounding these cells may communicate *via* gap junctions (Pannese, 1981). Because there are so many changes that occur after injury, it is challenging to determine which mechanisms are essential contributors to the development of pain. It is also not yet understood why a portion of the SCI patient population do not report pain after similar injuries (Siddall and Loeser, 2001).

Synaptic changes

It is well established that binding of postsynaptic density protein-95 (PSD-95) to NMDA receptors participates in the downstream intracellular signaling events involved in synaptic plasticity (Christopherson et al., 1999; Kornau et al., 1995). Using the chronic constriction injury (CCI) model and histochemical staining, Garry *et al*. have shown that NMDA receptors form complexes with PSD-95 in the thoracic and lumbar spinal cord, and that PSD-95 expression is detected specifically in lamina II of the dorsal horn of the spinal cord (Garry et al., 2003). In this same study, they demonstrated that mutant mice expressing a truncated form of PSD-95 failed to develop NMDA receptor-dependent hyperalgesia and allodynia after a model of neuropathic pain (Garry et al., 2003). Additional work by Lu *et al*. revealed that neuronal activity and pain-related behaviors

associated with central sensitization could be altered by blocking the interaction between PSD-95 and the NR2B subunit of the NMDA receptor within the dorsal horn, as well as by lowering the interaction between PSD-95 and the multifunctional scaffold protein Kalirin-7 (Lu et al., 2015). This may suggest a role for PSD-95 and Kalirin in central sensitization and chronic pain. Earlier work using antisense oligonucleotides already showed that knockdown of PSD-95 in the spinal cord reduced behavioral hypersensitivity after nerve injury (D'Mello et al., 2011; LeBlanc et al., 2010; Tao et al., 2001).

It is apparent that NMDA receptors are critical in the progression of central neuronal hyperexcitability (Coderre et al., 1993; Eide, 1998; Hara and Snyder, 2007). Inhibition of NMDA receptors further supports the observation of the contribution of NMDA in the development of central neuropathic pain after SCI (Eide et al., 1995; Inquimbert et al., 2018; Zhou et al., 2011). Additional work indicates the involvement of glutamate in central sensitization as an activator of AMPA receptors in the spinal cord, producing an influx of calcium to cells and irreversible damage following injury. AMPA receptors are also expressed in oligodendrocytes and astrocytes, suggesting a role for glutamate in non-neuronal subtypes and their possible involvement in chronic pain (Latremoliere and Woolf, 2009; Li and Stys, 2000).

Changes in gene expression

The net result of physical tissue damage, inflammation, and central sensitization after injury is an increase in neuronal activity. This hyperexcitability leads to changes in gene expression and consequently even more changes throughout the nervous system. It is entirely possible that neuropathic pain is associated with major changes in gene expression in primary sensory neurons that innervate areas near or at the site of injury in the spinal cord.

Using non-SCI models of injury, studies have shown that specific subtypes of hypersensitivity temporally diverge based on differences in gene expression in sensory neurons; for example, nociceptor transcripts initially change with the onset of cold allodynia, while immune cell transcripts change more slowly as tactile hypersensitivity develops (Cobos et al., 2018). Although we now know that target-derived growth factors are essential for development and cell survival, research has established that they also have a role in regulating everyday functional properties of sensory neurons in the adult (Albers et al., 2006; Stucky et al., 1999). Following injury, inflammation triggers an increase in target-derived growth factors, whereas after peripheral axon damage there is a decrease (Mannion et al., 1999). Both changes produce alterations in the levels of neurotransmitters, ion channels, receptors, and structural proteins (Shu and Mendell, 1999). While inflammation may not be sufficient to cause neuropathic pain, chronic inflammation prompts sensory neuron gene expression modification, including changes in genes involved in ion channel expression (Woolf and Costigan, 1999). Acute sensory neuron sensitization occurs locally, at the site of injury, but long-term sensory changes are dependent on transcriptional changes of ion channels at the cell body in the DRG (Mao et al., 2004; Woolf and Costigan, 1999). These changes occur post-inflammation, outside of the acute phase of injury, due to the inherent delay of changes in gene expression and protein transport (Linley et al., 2010). Increased expression of retrograde trafficking of pro-nociceptive molecules such as substance P, CGRP, NGF, and GDNF also results from these changes. This can induce changes in membrane expression of ion channels that increase the excitability of sensory neurons by lowering their sensory thresholds (Boucher et al., 2000; Cheng and Ji, 2008; Cummins et al., 2000). In addition to retrograde signaling of growth factors, increased electrical activity generated by calcium influx through voltage-gated ion channels in the spinal cord can also cause transcriptional changes in sensory neurons (Woolf and Salter, 2000).

Early studies established the importance of NGF, when mice lacking the growth factor or its receptor TrkA resulted in mice lacking nociceptors (Crowley et al., 1994; Malin et al., 2006; Silos-Santiago et al., 1995). In humans, loss-of-function mutations in the *NTRK1* gene (encoding TrkA) manifest as congenital insensitivity to pain syndrome (Indo, 2001; Shatzky et al., 2000). Injection of NGF produces thermal and mechanical hypersensitivity in both rodents and humans, although over differing time courses (Malin et al., 2006). NGF-TrkA interaction on the peptidergic class of nociceptors activates downstream signaling pathways, such as phospholipase C (PLC), mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K) by increasing intracellular free calcium levels within sensory neurons. This activates TRPV1, producing a change in heat sensitivity, and orchestrates further changes in transcription, translation and post-translational modification of sensory neuron ion channels (Chuang et al., 2001). NGF can also be transported in a retrograde direction to the cell body of nociceptors in the DRG, where it can stimulate expression of substance P, TRPV1, and $Na_v1.8$ voltage-gated sodium channels (Chao, 2003; Ji et al., 2002). Kinase signaling serves two purposes: it allows relatively fast activity-dependent changes by regulating local protein levels and channel activities, while also altering long-term transcriptional changes (Linley et al., 2010). The cumulative outcome of changes in gene expression of NGF results in the amplification of neurogenic inflammation (Basbaum et al., 2009).

Primary sensory neurons expressing TrkB are also involved in pain signaling. This has been demonstrated following light touch after nerve injury in mice. Mice lacking TrkB display less sensitivity to touch and lack mechanical allodynia in a model of neuropathic pain. Consistent with this observation, activation of TrkB-expressing neurons produces nociceptive behavior in wildtype mice (Dhandapani et al., 2018). Additionally, studies have shown that cellular immediate-early genes, such as c-fos, are expressed in spinal cord neurons following inflammation and the activation of nociceptors, suggesting rapid activation of genes and changes in transcription after injury (Dubner and Ruda, 1992). It is evident that following injury there is an increased expression

of sodium channels on damaged C-fibers, as well as the release of growth factors, all of which further trigger changes in channel and receptor expression on both injured and uninjured fibers, and perpetuate the perception of pain (Baron, 2006).

Supraspinal alterations

While the primary focus of SCI is at or near the site of injury, it is important to discuss changes in the surpraspinal pathway and its involvement in neuropathic pain. Primary afferents communicate noxious information from the periphery to projection neurons within the dorsal horn of the spinal cord. A subset of these projection neurons convey information to the thalamus and finally to the somatosensory cortex (Basbaum, 2000). Additional subsets of projection neurons communicate the affective component of pain, or act as a feedback system *via* connections in the rostral ventral medulla, midbrain periaqueductal gray, brainstem, and amygdala (Basbaum et al., 2009). There is evidence to support the idea that the development of persistent pain after SCI is dependent on a balance between nociceptive and non-nociceptive sensory inputs at the thalamic level (Eide, 1998). Neurosurgical studies have found a correlation between neurons in the somatosensory thalamus of patients reporting neuropathic pain and an increase in spontaneous firing rates and evoked responses not normally capable of activating those neurons. This has also been observed in patients with spinal cord transections and subsequent neuronal hyperactivity in thalamic regions denervated of their normal sensory afferent input (Coderre et al., 1993). Another study utilized transcriptome analysis using publicly available databases to show that certain pathways were enriched in the brain following SCI (Baek et al., 2017). These included oxidative phosphorylation, inflammatory response pathways, and endoplasmic reticulum stress-related pathways, among several others. Different pathways were activated at different time periods after injury, suggesting differences in gene expression patterns at acute (3 hours post-injury) and sub-acute (2 weeks post-injury) phases in the brain (Baek et al., 2017).

Animal models of SCI

Models of SCI-induced neuropathic pain focus primarily on injury caused by contusion or weight drop, spinal cord compression, transection, excitotoxic lesions, or ischemic injury (Marques et al., 2009; Siddall and Loeser, 2001) (Table 1-1). Although several models have been developed to better understand the contribution of distinct injury models to the initiation of pain, there is no consensus as to the best model to use for SCI pain research. Given the diversity of human injuries, all of the current animal models have face validity. A practical approach is to utilize several different injury models to find common features that provide more realistic therapeutics that will work for the majority of SCI patients (Gaudet et al., 2017; Kjell and Olson, 2016; Shiao and Lee-Kubli, 2018).

Table 1-1. Summary of the diverse models used to study SCI.

Model	Method	Animal	Level of injury	Study
Compression	Clip-compression	Rat, mouse	C6, T3, T4, T9, T12,	(Bruce et al., 2002; Forgione et al., 2017; Joshi and Fehlings, 2002a, b; Marques et al., 2014; Marques et al., 2009; Tator, 2008; Ziu et al., 2014)
Contusion	Impactor	Rat, mouse	T8, T9, T13	(Basso et al., 2006; Farooque, 2000; Gaudet et al., 2017; Kuhn and Wrathall, 1998; Ma et al., 2001; Wrathall et al., 1985)
Transection	Vanna spring scissors, micro scalpel	Rat, mouse	T9, T10	(Lukovic et al., 2015; Seitz et al., 2002; Ung et al., 2008)
Excitotoxic	Glutamate, quisqualate, 3-morpholinosydnonimine (SIN-1)	Rat, mouse	T13, L3,	(Bao et al., 2003; Fairbanks et al., 2000; Liu et al., 1999)
Ischemic	Irradiation, aortic occlusion	Rat, rabbit	T8, T10	(Celik et al., 2002; Giulian and Robertson, 1990; Hao et al., 1991; Jaggi et al., 2011; Watson et al., 1986)

Conclusion

While multiple SCI models exist, assessment of pain remains a challenge. Although insight into changes of the nociceptive system has improved, the mechanisms underlying the transition from acute to chronic pain have yet to be resolved. Such pain is particularly difficult to assess in both humans and rodents because a primary feature is spontaneous pain (Finnerup, 2017). Outcome measures in rodent models rely on spinally mediated withdrawal reflexes and assessment of motor activity. These models have a very limited response repertoire compared to human patients, which is further confounded by the differences in the rate and extent of SCI recovery between rodents and humans (Costigan et al., 2009; Lankhorst et al., 2001). A more advantageous approach to circumvent this issue may be to study underlying genetic changes rather than phenotypic responses in rodent models. More recently, an emphasis has been placed on the concept of “time is spine”, which highlights early interventions to improve long-term outcomes (Hachem et al., 2017). “Time is spine” concentrates on rapid transfer of patients to centers specializing in spinal cord injuries, improving early surgery to accomplish spinal cord decompression, and encouraging additional treatments with proven long-term benefits, such as steroid treatments and blood pressure stabilization. Irrespective of injury model, SCI alters genetic, cellular, and molecular pathways that all contribute to the development of neuropathic pain. Because of this, a multifaceted approach is necessary to better understand mechanisms of SCI pain and for the development of new treatment strategies and models.

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GLOSSARY

- *Allodynia:* Painful response to a normally non-noxious stimuli.
- *Cytokines:* Small proteins secreted by cells of the immune system that are involved in the induction of inflammatory responses. Cytokines include chemokines, interferons, and interleukins.
- *Dermatome:* An area of skin supplied by a single spinal nerve.
- *Dysesthesia:* Unpleasant or abnormal sense of touch.
- *Erythromelalgia:* Intense, burning pain, redness (erythema), swelling, and increased skin temperature that primarily affects the extremities.
- *Hyperalgesia:* Increased sensitivity to already painful stimuli.
- *Neuropathic pain:* Pain caused by a lesion or disease of the peripheral or central nervous system.
- *Nociceptor:* Sensory neuron that transduces painful stimuli into transmitted neuronal signals.
- *Pro-inflammatory state:* Period in which cytokines that induce inflammation are released, in addition to the release of reactive oxygen species and ATP, all of which results in a harsh environment that supports inflammation.
- *Wallerian degeneration:* The degeneration of nerve fibers that occurs after injury or disease of the PNS or CNS that begins at the site of injury and continues distal to the site of injury, while the cell body remains intact.

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CHAPTER 2

The pathogenesis of pain following Spinal Cord injury: Recruitment of afferent nociceptors distal to the site of injury

Introduction

Persistent pain following spinal cord injury (SCI) is an adverse consequence of neural injury and is a debilitating concern for most patients (Costigan et al., 2009; Walters, 2012). In many disorders, pain is a symptom that dissipates following appropriate treatment of the underlying pathology. However, in conditions such as SCI, the underlying issue cannot always be cured, and pain becomes the patient's primary concern (Baron, 2006). Previous studies have demonstrated that SCI increases afferent excitability, driving peripheral neurogenic inflammation and amplifying input to the spinal cord (Wu et al., 2013). Extensive work has characterized how spinally mediated alterations contribute to SCI-induced pain. Studies have established a variety of factors that impact how incoming sensory stimulation is processed, all of which increase excitability within the spinal cord (Bruce et al., 2002; Meisner et al., 2010; You et al., 2008). Blockade of afferent input into the central nervous system (CNS) can effectively mitigate discomfort and a multitude of central changes following chronic pain, supporting the idea that the mechanisms generating and maintaining these prolonged pain states reside within the peripheral nervous system (PNS) (Basbaum et al., 2009; Campbell et al., 1988; Gold and Gebhart, 2010). Accordingly, specialized afferents, known as nociceptors, also contribute to the generation and persistence of SCI pain (Walters, 2012).

Hypersensitivity following centrally generated pain can generally be categorized by the region affected: above-level pain occurring in cranial tissue, at-level pain occurring at or near the site of injury, and below-level pain localized caudal to the injury site (Baron, 2006; Carlton, 2011). These pain abnormalities are clinically characterized by spontaneous pain, occurring in the absence of peripheral stimulus, or as evoked pain. Evoked pain can be exhibited as allodynia, where non-noxious stimulus is perceived as noxious, or as hyperalgesia, where a noxious stimulus produces an amplified pain response (Carlton, 2011). Increased behavioral responding is generally elicited by increased neuronal output from unmyelinated C-fiber afferents and thinly myelinated Aδ

primary afferents, which are normally silent in the absence of noxious stimuli. The somatosensory system is organized so that specialized sensory neurons encoding low intensity stimuli specifically activate proprioceptive pathways, whereas high intensity stimuli only activate nociceptors that lead to pain. Under non-injured conditions, these two pathways do not overlap (Woolf, 2011). However, changes in sensory processing following injury results in central sensitization, where these two previously parallel pathways now functionally intersect (Woolf, 2011). The reorganization that occurs following injury increases the amplitude and duration of pain signals, strengthens synapses, and reduces inhibition, resulting in a distortion or enhancement of pain processing.

Nociceptors are specialized sensory organs that selectively respond to noxious stimulation of the skin, muscle, joints and viscera. The cell bodies of nociceptors are located in the dorsal root ganglia (DRG) and have a peripheral and central axonal projection that innervates a target organ and the dorsal horn of the spinal cord, respectively (Basbaum et al., 2009). Following injury, nociceptors sensitize, exhibiting a decrease in activation threshold and an increase in firing rate (Carlton, 2011). After SCI, these sensitized nociceptors increase input into the spinal dorsal horn, and initiate an injury-induced state of excitability. However, it is still unclear how afferents that are not at the site of injury become sensitized. Research has demonstrated that cell bodies within dorsal root ganglia (DRG) several segments above and below the level of injury also display altered firing characteristics in response to SCI, exhibiting spontaneous activity (SA), which is thought to underlie persistent, ongoing pain (Carlton et al., 2009; Carlton, 2011). This raises the question of the role of nociceptors in SCI and their contribution to the induction and maintenance of pain following injury.

Recent studies suggest that SCI and the subsequent injury-related processes impact primary sensory neurons. Each sensory neuron has a unique pattern of gene expression that influences

the modality-specific contribution of nociceptors to injury-induced pain. Currently, there are few studies that examine the differential function of specific afferent subtypes in response to SCI. Of particular interest are the heterogeneous population of C-fibers that are classified by the presence or absence of neuropeptides. The molecular and physiological identity of nociceptors has not been fully elucidated, but it is known that these specific afferents are vulnerable to spinal insult and play a significant role in driving injury-induced pain. Each nociceptor subtype can be characterized by different patterns of protein and gene expression. Peptidergic nociceptors express calcitonin gene-related peptide (CGRP), substance P (SP), and the TRPV1 receptor, while the nonpeptidergic population binds isolectin-B4 (IB4), and expresses MrgprD (Lawson et al., 2008). Nonpeptidergic afferents also express the Ret receptor, which is targeted by glial-derived neurotrophic factor (GDNF) family ligands (Basbaum et al., 2009). The GDNF family of ligands include GDNF, neurturin, artemin, and persephin, which bind to their respective receptors; GFRα-1, GFRα-2, GFRα-3, and GFRα-4. GFRα-1-3 are expressed by peripheral neuronal populations and may contribute to nociceptor sensitivity (Karczewski et al., 2010; Lingueglia, 2007). Protons also modulate a variety of receptors and ion channels expressed in sensory neurons, and acid sensing ion channel 3 (ASIC3) has been implicated in both mechanical and thermal changes following injury (Deval et al., 2010; Jankowski et al., 2009).

Few studies have examined the role of afferents, which are likely contributors in the generation of pain, following SCI. These afferents may function as an ideal site for intervention as they contribute to hypersensitivity following injury, and are located in the periphery where they are easily accessible for treatment administration. However, it is still unclear how afferents distal to the site of injury become sensitized. The following experiments begin to address how afferent fibers change following SCI by characterizing transcriptional changes, and correlating these changes to behavioral phenotypes. Determining molecular organization of subtypes helps us to better understand the contribution to normal function as well as the underlying pathophysiology

of hypersensitivity following SCI. A better understanding of the function of subsets of primary sensory neurons is fundamental to elucidate specific potential targets for pain treatment.

Results

Selective alterations in gene expression after sham surgery

We first examined gene expression changes in whole tissue from sham mice where a laminectomy at T10 was performed. The target gene panel for each tissue differs based on gene expression specific to that tissue. As a result, the full group of specific targets were not analyzed for each tissue type, however all possible targets that were assessed are included in each graph. It is likely that the removal of bone and muscle alone may result in acute, as well as long-term, gene expression changes after surgery (Macrae, 2008; Sansone et al., 2015). In order to identify what changes occurred due to injury from the laminectomy and not to the spinal cord, we compared whole tissue gene expression between sham and naïve mice. To assess potential changes occurring in the periphery after surgery, we looked at alterations in hindpaw hairy skin. Gfrα2 and Trpv1 are significantly upregulated 7 days following laminectomy in comparison to naïve levels (**Fig. 2-1A**), and Trpv1 is significantly increased at 7 days in comparison to 24hr following surgery (**Fig. 2-1A**). We also examined changes in the quadriceps muscle to further assess changes that may occur below the level of injury in the periphery after laminectomy. Most transcript changes occurred 7 days post-surgery; Asic2, Calc-α, P2rx3, Trka, Trpa1 (**Fig. 2-1B)** are significantly different from naïve transcript levels**.** P2rx3 is also significantly different between 24hr and 7 days post-surgery (**Fig. 2-1B**). Additional experiments performed at a third time point (4 days) post-surgery exhibited similar changes in transcripts Calc-α and P2rx3, indicating the potential importance of these two genes at multiple time points following laminectomy (**Chapter 3**). This is in contrast to several other transcripts listed in Fig. 2-1 that may have greater involvement 7 days post-surgery but not at earlier time points, such as 4 days.

While the spinal cord is left untouched during sham surgery, removal of bone and muscle surrounding the cord exposes the tissue to a new environment. Because of this, we also assessed changes in the spinal cord tissue at the level of laminectomy (T9, T10, T11) and below the level of laminectomy (L1, L2, L3). We found no significant changes at either time point at the level of laminectomy (**Fig. 2-1C**). However, Trpv1 was significantly upregulated 7 days post-surgery in comparison to both naïve and 24hr transcript levels (**Fig. 2-1D**). Although we did not predict to observe such large changes in comparisons between sham and naïve mice after surgery, the removal of bone and muscle alone could produce post-surgical pain analogous to what has been reported in humans (Woolf, 2011). This was taken into consideration when we went on to examine transcript changes that occurred post-SCI, and therefore all transcripts were normalized to sham mice instead of naïve mice.

Changes in the periphery are likely due to changes occurring in the DRG at or around the site of injury (Ferrari et al., 2013; Yang et al., 2014). Because we observed significant changes in transcript levels in the skin and the muscle below the level of injury, we also inspected transcript levels in DRG at (T9, T10, T11) and below (L1, L2, L3) the level of laminectomy. We observed a significant decrease in Asic3 and Gfrα2 in comparison to naïve tissue 7 days after injury. Gfrα2 transcript levels are also significantly down-regulated in comparison to levels 24hr following laminectomy, as well as Gfrα1 levels (**Fig. 2-1E**). We observed similar changes below the level of laminectomy; Gfrα1 expression is significantly decreased at 7 days in comparison to transcript levels at 24hr and Gfrα3 levels are significantly decreased at 7 days in comparison to naïve levels.

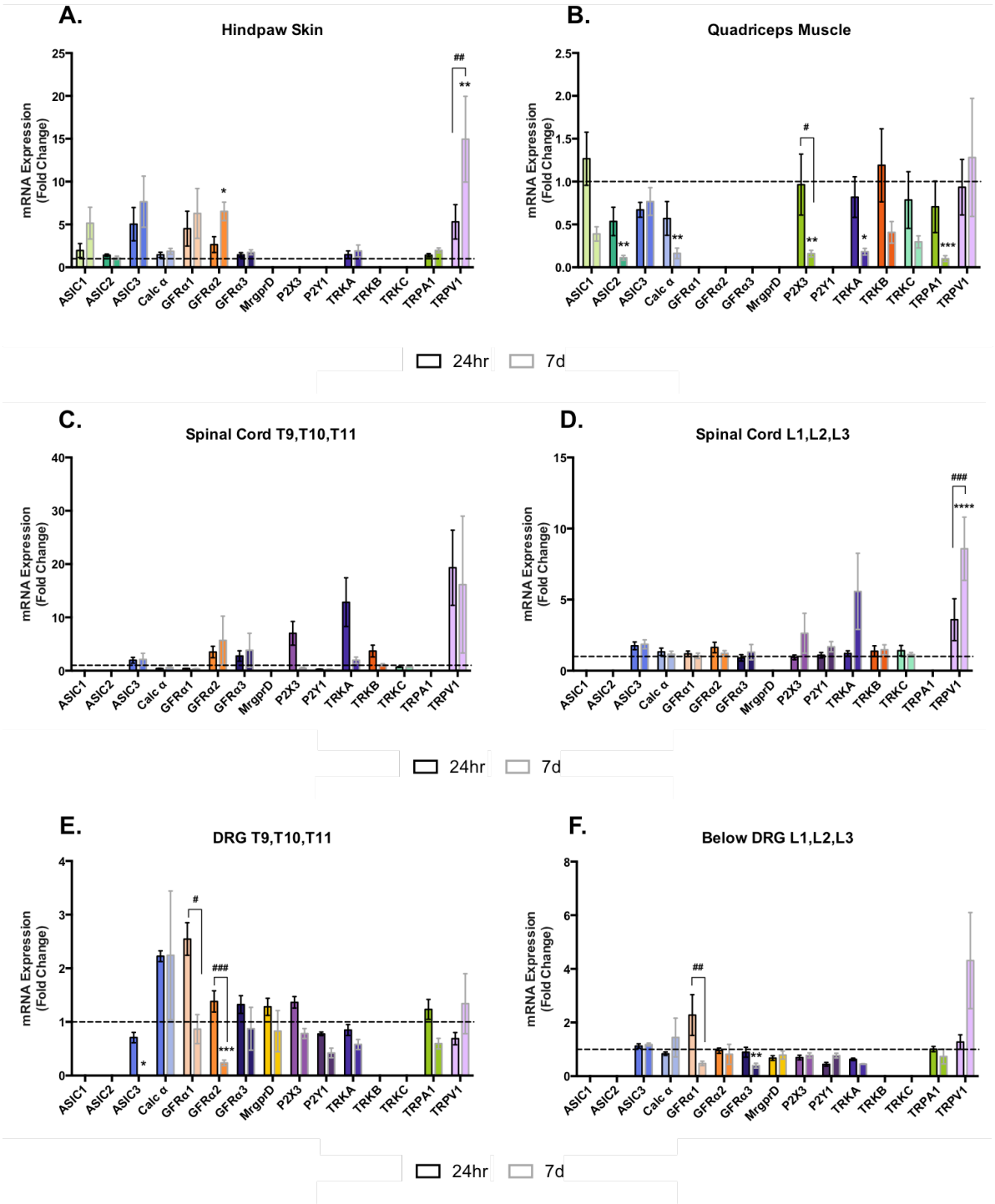

Figure 2-1. Hindpaw hairy skin (A), quadriceps muscle (B), spinal cord segment and DRG at the level of laminectomy (C,E), spinal cord segment and DRG below the level of laminectomy (D,F) collected from mice 24hr or 7d following laminectomy, normalized to naïve tissue. N=4-7 mice, *p < 0.05; **p < 0.005; ***p < 0.001. Asterisks and pound signs indicate the results of Tukey's tests following 2-way ANOVAs. * Represents significant differences between sham and naïve mice, # corresponds to significant differences between sham time points, error bars indicate SEM.

SCI-induced injury correlates with changes in pain related target gene expression

We next used the same panel of gene targets to examine transcript changes in receptors during the acute phase of injury at the whole tissue level. We collected tissue from spinally injured mice 24hr and 7 days following SCI to capture the acute phase of injury at two different injury severities (70kD, 10s dwell or 65kD, 1s dwell). The hairy hindpaw skin and quadriceps muscle were assessed as potentially affected below-level tissue in the periphery. Spinal cord segments at (T9, T10, T11) and below (L1, L2, L3) the site of injury were collected, as well as corresponding DRG. All tissue (24hr or 7d) was normalized to sham tissue that corresponded to each time point (24hr or 7d, respectively).

Hindpaw hairy skin showed significant down regulation of Asic3 between low and high injury severity at 24hr (**Fig. 2-2A**). At 7 days post injury, Asic1, Asic3, Calcα, Gfrα1, Gfrα2, Trka, Trpa1, Trpv1 are all significantly down regulated in comparison to 7d sham controls following the lower severity injury. Asic3, Gfrα2, Trka, and Trpv1 transcript levels are also significantly decreased 7 days following the higher injury severity. Calcα, Trpa1, and Trpv1 differ significantly between the two different injury severities.

We observed a significant difference between injury severity levels in quadriceps muscle 24hr post-injury in Asic1 transcript levels (**Fig. 2-2B**). Further changes were detected at 7 days post injury in quadriceps muscle between injury severity levels in Trka mRNA and Trpv1 levels. Asic1, Asic2, Calcα, P2rx3, Trka, Trkb, and Trpv1 are all significantly up regulated after receiving 65kD, 1s dwell injury. Changes in Asic1 transcript are also observed at the higher severity injury level.

While we predicted to see changes in transcript levels below the level of injury, we also anticipated to observe significant alterations in transcript levels in spinal cord tissue itself following injury. At 24hr post-SCI (65kD, 1s dwell injury severity), at the level of injury, we observed a down regulation

in Gfrα2, Gfrα3, P2rx3, P2ry1, Trka, Trkb, and Trpv1 (**Fig. 2-2C**). We did not observe significant changes at the same time point in mice that received the higher injury severity (70kD, 10 s dwell). However, there are significant differences between the two injury severities in transcript levels of P2rx3 and P2ry1. We also assessed changes in gene expression in the spinal cord at the level of injury 7 days post-SCI. Asic3 levels were significantly down regulated at the lower injury severity level, and are significantly different from the higher severity injury. Gfrα3 and Trpv1 levels also differed significantly between the two injury severity conditions, while Trpv1 mRNA levels are significantly down regulated at the higher injury severity.

Because we observed significant changes at the level of injury in the spinal cord, as well as in peripheral tissues below the level of injury, we also wanted to assess changes in the spinal cord directly below the level of injury at L1, L2, and L3. However, we did not observe any significant changes in the spinal cord at these levels 24hr post-SCI in either injury severity or between injury severities (**Fig. 2-2D**). We also did not detect any significant changes at 7 days post-injury.

The DRG also play a major role in nociceptive signaling after injury, and so we assessed changes in mRNA levels in a similar gene panel in the DRG corresponding to where the SCI was conducted (T9, T10, T11). Mrgprd transcript levels significantly differ between the two injury severity conditions 24hr post-injury (**Fig. 2-2E**). P2ry1 and Trpa1 mRNA levels are significantly increased 7 days after SCI in mice that received the higher severity injury. The two injury severity conditions differ significantly in P2ry1 and Trpv1 mRNA levels.

Although we did not identify any significant changes in transcript levels in our chosen gene panel in the spinal cord below the level of injury, we wanted to investigate if there were any changes in the corresponding DRG, as we did observe changes in below-level peripheral tissues to which these DRG project. We did observe significant differences in P2ry1 and Trpv1 gene expression

24hr post-SCI in mice that received the higher injury severity (**Fig. 2-2F**). Trpv1 also differs significantly between the two injury severity conditions. We also assessed changes 7 days after injury, and observed a significant difference in Calcα, Gfrα1, Gfrα3, Trpa1 and Trpv1 in mice that received 75kD, 10s dwell compression injury. Gfrα3 transcript levels are significantly upregulated, while Trpv1 levels are significantly decreased in mice that received the lower injury severity compression injury. The two injury severity conditions also differ significantly in expression of Calcα, P2rx3, and P2ry1. However, additional studies that examined changes in DRG below the level of injury 4 days post-SCI did not display significant changes in these transcripts (**Chapter 3**), suggesting that these transcripts may play an important role at specific time points in their contribution to acute, or the transition from acute to chronic, pain.

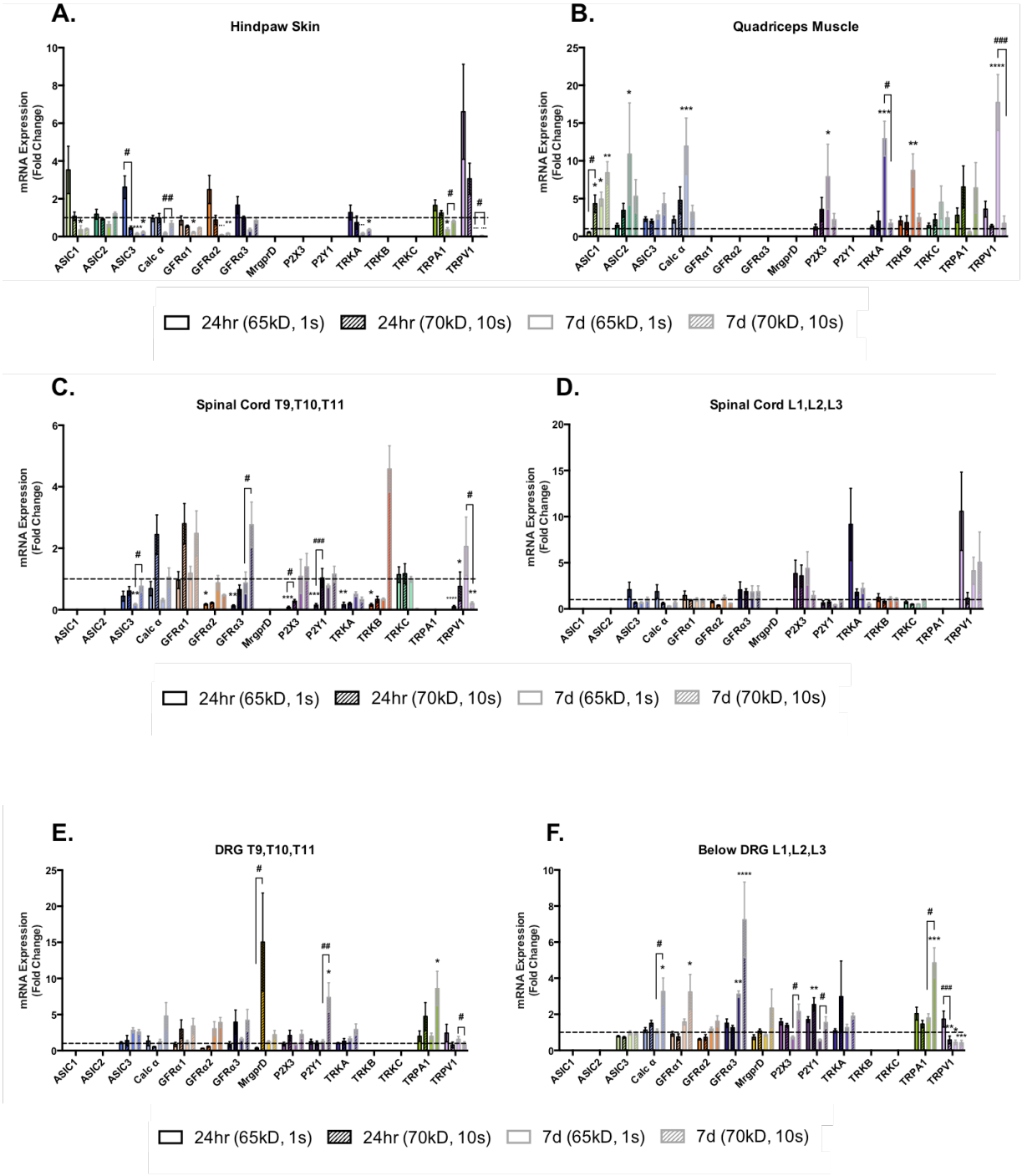

Figure 2-2 Hindpaw hairy skin (A), quadriceps muscle (B), spinal cord segment and DRG at the level of laminectomy (C,E), spinal cord segment and DRG below the level of laminectomy (D,F) collected from mice 24hr or 7d following moderate or severe SCI, normalized to sham tissue. N=4-7 mice, *p < 0.05; **p < 0.005; ***p < 0.001 Asterisks and pound signs indicate the results of Tukey's tests following 2-way ANOVAs. * Represents significant differences between sham and naïve, # corresponds to significant differences between sham time points, error bars indicate SEM.

Backlabeling of the saphenous nerve as a tool to selectively label nociceptor subpopulations that project to the skin

Each sensory neuron has a unique pattern of gene expression that influences the modality-specific contribution of a nociceptor to injury-induced pain. After examining whole tissue transcript changes, we next wanted to examine the differential expression profiles of specific nociceptor populations in response to SCI, specifically in the skin, to better understand how gene expression within individual neurons impacts nociceptor function and how this may contribute to the behavioral hypersensitivity that is observed below the level of injury following SCI. To address this we backlabeled the saphenous nerve, projecting from the hindpaw hairy skin to L2, L3 DRG, with wheat germ agglutinin (WGA)-488 or IB4-488 to target a general population of cutaneous afferents and nonpeptidergic cells, respectively. We validated backlabeling of both WGA-488 and IB4-488 conjugated dyes via the saphenous nerve at 2 or 3 days subsequent to nerve injection, and confirmed RNA extraction and amplification from a single cell by gel electrophoresis of PCR product as well as confirming proper detection and amplification during PCR (**Fig. 2-3 A-D)**.

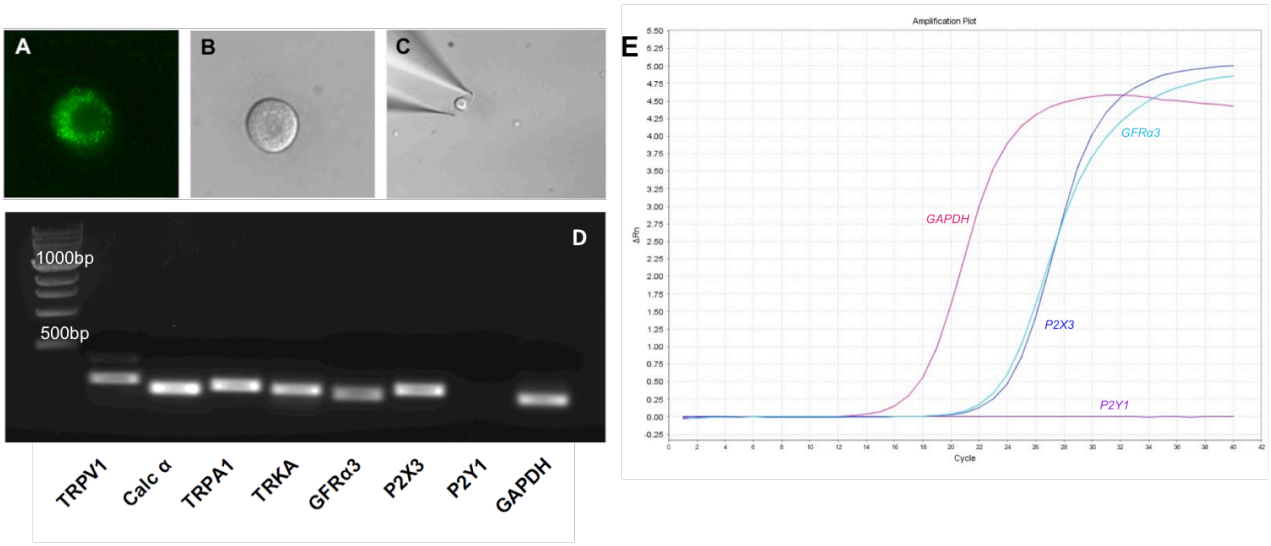

Figure 2-3. Backlabeled cell seen under fluorescence (A) and brightfield illumination (B). Representative image of collection of a single cell (C). RT-PCR gene expression profile (D) and amplification plot (E) from a WGA backlabeled cell following 24hr SCI expressing peptidergic markers including Trpa1, Trpv1, and Calcα.

Sham surgery correlates with specific gene expression changes in nociceptors that project to the skin

To determine changes that occurred in response to the removal of bone and muscle during the laminectomy and not to changes from injury to the spinal cord, we first assessed gene expression levels in sham versus naïve mice at 24hr following surgery. We found significant differences in Asic1 gene expression in sham mice backlabeled with WGA (**Fig. 2-4A**). We also observed significant transcript changes in Gfrα3 in sham mice backlabeled with IB4 (**Fig. 2-4B**). Additional work that examined transcriptional changes between sham and naïve mice 4 days post-surgery in a nociceptor-enriched cell population exhibited a significant decrease in Gfrα3 as well (**Chapter 3**). Although we did not see comparable changes with Asic1, this may be indicative of the distinct roles different transcripts may play at varying time points.

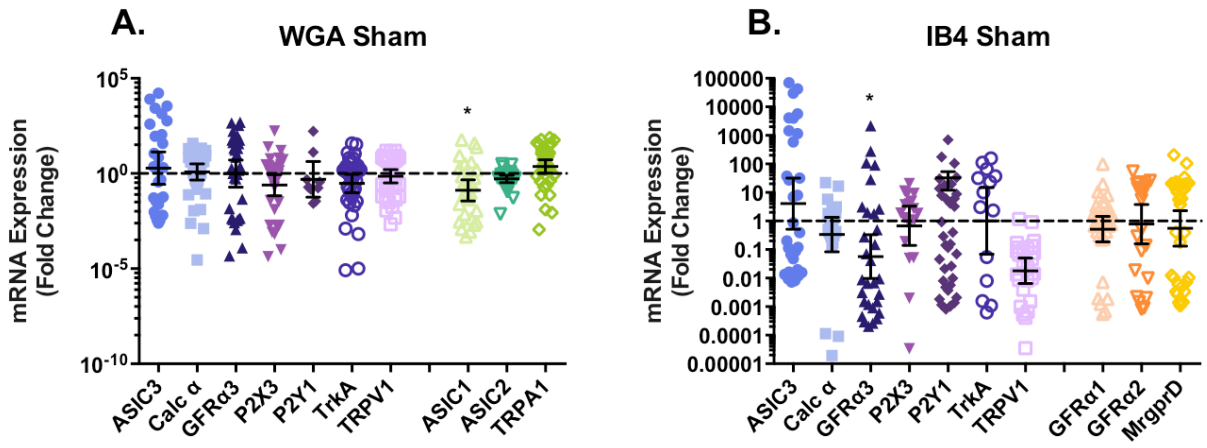

Figure 2-4 Real-time RT-PCR performed on individual cutaneous afferents backlabeled from the saphenous nerve from mice that received sham surgery. Single cell mRNA expression shows log-scale distribution among genes of interest. Cells were backlabeled with the marker WGA-488 (A) or the non-peptidergic specific marker IB4-488 (B). Individual neurons were collected 24hr following sham surgery. Asic1 mRNA levels are significantly decreased in the WGA population (Students unpaired t-test, p=0.0285) (A) and Gfrα3 is significantly decreased in the IB4 population (Students unpaired t-test, p=0.0065) (B). Data normalized to naïve controls (dashed line) and graphed as fold change. Statistics were performed on ΔCT values, N=4 mice/condition, 20-40 cells/ gene target. *p < 0.05; **p < 0.005, * indicates significant change in comparison to naïve controls, error bars indicate SEM.

SCI-induced injury correlates with changes in nociceptors that project to the skin

WGA-488 binds to N-acetylglucosamine and N-acetylneuraminic acid (sialic acid) residues on cell membranes, and can be used as a retrograde tracer to non-selectively label neurons. We utilized this dye to label both peptidergic and non-peptidergic populations of nociceptors that project via the saphenous nerve to the hairy hindpaw skin. Mice received an injection of WGA-488 into the saphenous nerve 2 days prior to single cell collection, and 1 day prior to SCI. After receiving the higher injury severity (70kD, 10s dwell) mice exhibit significant differences at the single cell level in Trpa1 (**Fig. 2-5A**). We also used IB4-488 to selectively bind the non-peptidergic population of neurons that project to the saphenous nerve, 3 days prior to single cell collection, and 2 days prior to SCI. After receiving the higher injury severity condition, mice exhibit significant differences at the single cell level in Asic3 (**Fig. 2-5B**).

We also wanted to consider changes that may be occurring at the single cell level in the lower injury severity condition (65kD, 1s dwell). We observed a significant upregulation of Trka mRNA expression (**Fig. 2-5C**) and a significant decrease in Trpa1 expression in cells labeled with WGA. We also found a significant increase in Gfrα3 in sham mice backlabeled with IB4 (**Fig. 2-5D**).

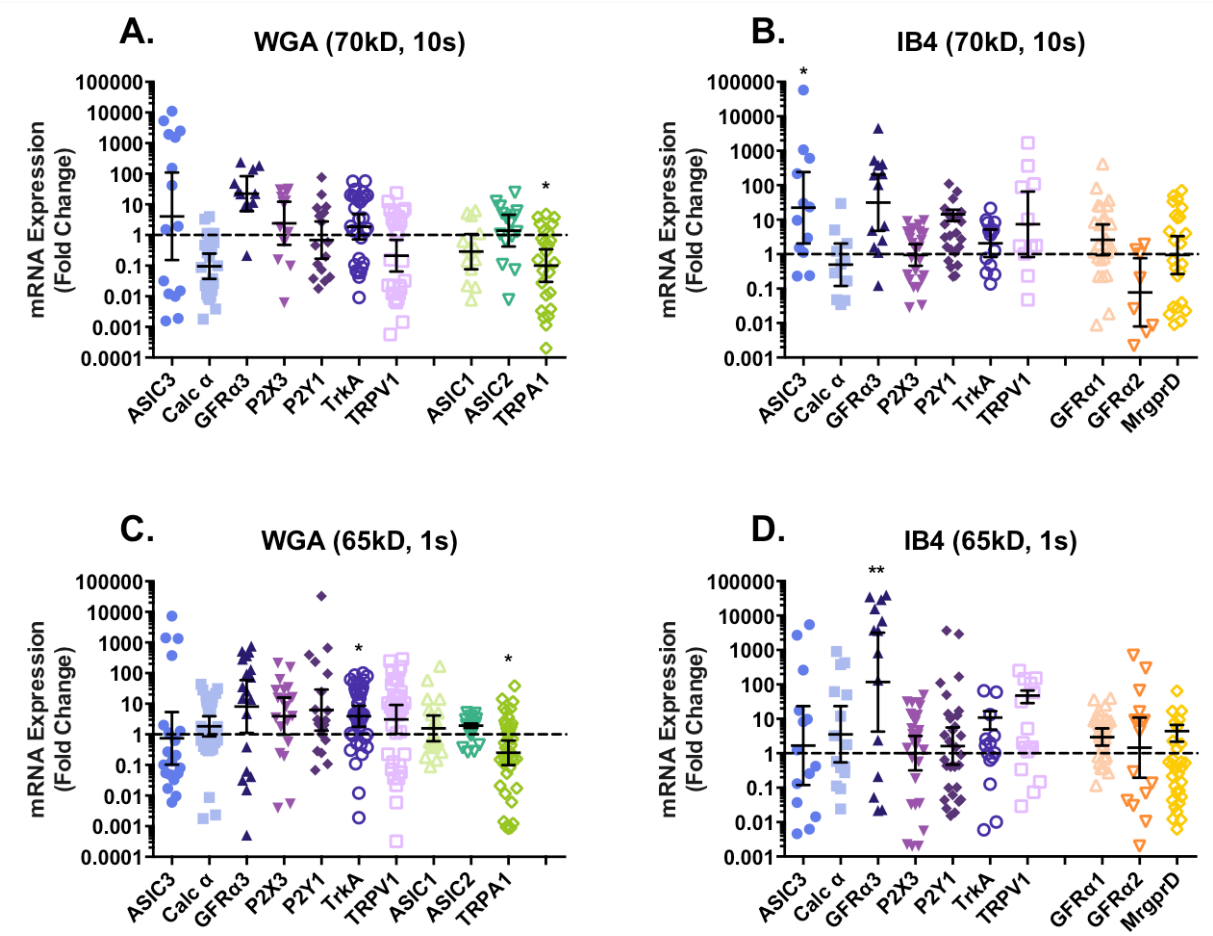

Figure 2-5 Real-time RT-PCR performed on individual cutaneous afferents backlabeled from the saphenous nerve from mice that received SCI. Cells were backlabeled with the marker WGA-488 (A,C) or the non-peptidergic specific marker IB4-488 (B,D). Individual neurons were collected 24hr following SCI at two different injury severities. Trpa1 is significantly decreased following the higher severity SCI (Students unpaired t-test, p=0.0473) (A) and Asic3 is significantly upregulated in the IB4 population (Students unpaired t-test, p=0.0479) (B). At the lower severity injury Trka mRNA expression is significantly increased and Trpa1 is significantly decreased (Students unpaired t-test, p=0.0456, p=0.0252 respectively) (C). Gfrα3 is significantly increased in the IB4 population (unpaired t-test, p=0.0065) (D). Data normalized to sham controls (dashed line) and graphed as fold change. Statistics were performed on ΔCT values, N=4 mice/condition, 20-40 cells/ gene target. *p < 0.05; **p < 0.005, * indicates significant change in comparison to sham controls, error bars indicate SEM.

SCI-induced mechanical and thermal behavioral hypersensitivity is not evident during the acute period following injury

Persistent activity from afferent fibers contributes to the development and maintenance of chronic pain following SCI, and can be observed by the manifestation of mechanical (frequently used as a measure of allodynia) and thermal (frequently used as a measure of hyperalgesia) behavioral hypersensitivity (Bedi et al., 2010). To observe potential behavioral alterations during the acute stage of injury, we assessed mechanical sensitivity using von Frey filaments, thermal sensitivity using the Hargreaves' apparatus, and locomotor impairment using the Basso Mouse Scale (BMS).

We did not observe significant changes in mechanical response thresholds 1 day post-surgery, in either the sham or SCI injury groups. Despite exhibiting paralysis immediately after SCI, injured mice still responded to von Frey filament stimulation on the plantar surface of the hindpaw 24hrs later. However, when tested 4 days post-injury, injured mice demonstrate a significant decrease in response to mechanical stimulation that persisted at all time points until the duration of the behavioral testing at 28 days in comparison to naïve mice (**Fig. 2-6A**). SCI mice also differ significantly from sham treated mice at 4, 7, 14, and 21 days post-surgery.

We also assessed differences in thermal sensitivity of the tail. We did not observe significant differences between injured and naïve mice using the Hargreaves' test, however we did find a significant change in the sham treated response threshold 24 hrs post-laminectomy in comparison to both naïve mice and SCI mice (**Fig. 2-6B**). Sham mice also differ significantly from SCI mice on the final day of behavioral testing at 28d post-surgery.

To confirm locomotion deficits in mice following injury, we performed the Basso Mouse Scale at multiple time-points post-injury and observed significant recovery in locomotor performance by

day 14 (**Fig. 2-6C**) over the time course but did not observe full recovery (BMS score of 9) by the end of behavioral testing at day 28. It is not surprising to observe partial locomotor recovery in spinally injured mice over this duration of time, and our observations are consistent with what has been found previously in the literature, however the extent of recovery is dependent upon the severity of injury (Basso et al., 2006).

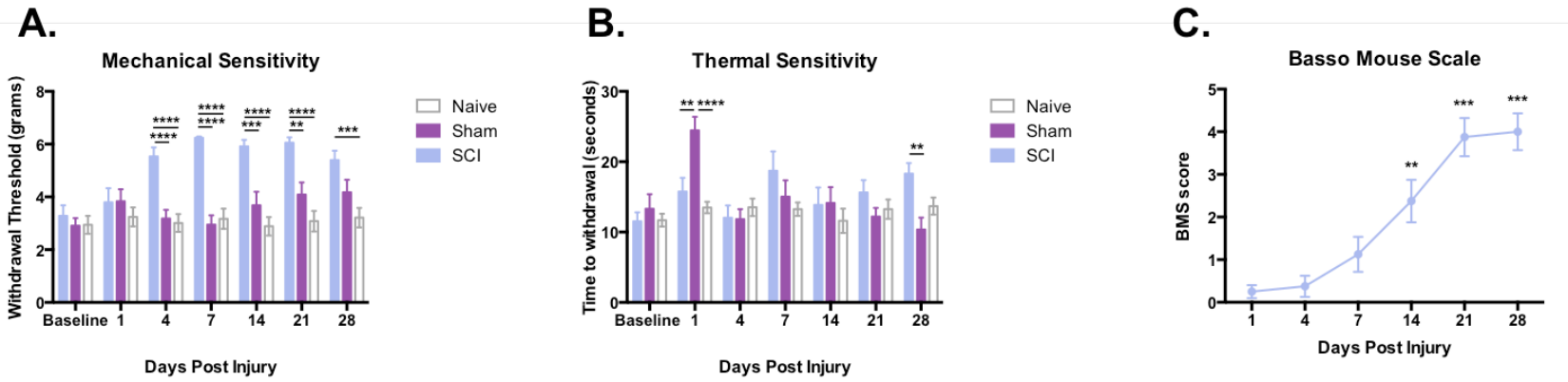

Figure 2-6 SCI mice show decreased responsiveness to mechanical stimulation following injury, while sham treated mice show no significant change (two-way ANOVA Tukey's multiple comparisons, $p<0.0001$ days 4, 7, 14, 21 and $p<0.001$ day 28) (A). Injured mice demonstrate slight reductions in tail flick latency following SCI, while sham mice show a decreased responsiveness 24hr following surgery but recover to naïve levels by day 4 (two-way ANOVA Tukey's multiple comparisons, $p<0.0001$ and $p<0.005$, respectively) (B). Basso mouse scale assay shows locomotive recovery in injured mice over a period of 28 days, however locomotive recovery is incomplete (one-way ANOVA Tukey's multiple comparisons, $p<0.05$) (C). N=6-8 mice/condition, *$p < 0.05$; **$p < 0.005$; ***$p < 0.001$, *** $p<0.0001$, * indicates significant recovery in comparison to day 1, error bars indicate SEM.

Discussion

Changes in whole tissue gene expression

The transition from acute to chronic pain is dependent in part upon functional plasticity at the molecular level along the nociceptive pathway (Kuner and Flor, 2017; Prescott et al., 2014; Sandkuhler, 2009). Following injury, gene expression is modified to support both biochemical and structural alterations that occur in order to support amplified pain signaling input, which results in the sensitization of nociceptive neurons (Khoutorsky and Price, 2018). Alterations in gene expression are also important for changes in nociceptive phenotypes, many of which do not originate at the cell body. It is likely that localized translation occurs at nociceptor endings and along injured axons to contribute to changes in gene expression, and this would account for rapid transcriptional and protein changes that occur immediately following injury (Ferrari et al., 2013). Because molecular changes typically reflect phenotypic characteristics, we anticipated significant changes at and below the site of injury in the spinal cord and DRG, as well as skin and muscle. Nociceptive terminals contain a variety of receptors associated with inflammation that are targeted at detection of molecular signals released following tissue injury.

Even in sham mice, we observed a significant increase in the expression of Gfrα2 and Trpv1 seven days post-surgery. Transient receptor potential vanilloid type 1 (TRPV1) is a member of the TRP family and plays a role in thermal sensitivity (Caterina et al., 2000). Previous work has shown that TRPV1 contributes to hypersensitivity that persists after SCI, and suggests a role for the contribution of TRPV1 generated spontaneous activity in nociceptors (Wu et al., 2013). It is noteworthy that Trpv1 decreases significantly at 7 days post injury following the 65kD, 1s dwell SCI, suggesting that the presence of Trpv1 after sham injury, but not after SCI, may be underlying a more acute pain phenotype in the skin. A significant increase of Trpv1 in the sham spinal cord, but a decrease in Trpv1 post-injury (at 24hr) would correspond with this observation. However, Trpv1 also increases in the spinal cord at the level of injury 7 days post injury, suggesting a time

dependent regulation of Trpv1 expression as well. Similarly, expression of Gfrα2 is significantly decreased at 7 days post injury in the skin of spinally injured mice and 24hr at the level of injury in SCI mice, but is increased in sham mice, suggesting deviating roles for these two genes in bone and muscle removal versus injury to the spinal cord. Translation of TRPV1 is driven in part by the neurotrophic factor, NGF, acting on tyrosine receptor kinase A (TRKA), which then phosphorylates intracellular stores of TRPV1 and ultimately results in insertion of TRPV1 into the cell membrane. An alternative explanation for the discrepancy between Trpv1 expression in sham versus injured mice is that following injury there is a limited supply of neurotrophic factors, including NGF, that are released from peripheral tissues following nerve degeneration and inflammation. A decrease in NGF signaling via p38 would result in decreased translation of Trpv1 in injured but not sham animals (Harriott and Gold, 2009). We also observed a significant decrease in Trka expression in SCI mice 7 days after injury in the skin as well as 24hrs post-injury at the level of injury in the spinal cord, which may further contribute to prevent expression of Trpv1 in the cell membrane during inflammation (Patapoutian et al., 2009). Neurotrophic signaling through TRK receptors regulates cell survival, proliferation, the expression of proteins such as ion channels and receptors, and synaptic strength and plasticity (Huang and Reichardt, 2003; Khoutorsky and Price, 2018). Consequently, a significant decrease in TRKA may have several downstream effects, which may account for some of the additional changes in gene expression that we observed. Time-dependent changes in the skin following SCI may also be caused by multiple sources. For instance, macrophage recruitment to inflamed skin below the level of SCI is suppressed from 2 to 5 days post-injury and may also influence changes in gene expression (Marbourg et al., 2017).

We also observed a decrease of Trka in the muscle at 7 days post injury in sham mice as well as in mice that received the 65kD, 1s dwell injury level. Some of the changes in muscle may be attributed to similar causes for gene expression change in the skin, as Trpv1 expression is also

decreased in the muscle of injured mice. Additionally, we observed a decrease in the expression of P2x purinoreceptor 3 (P2rx3) in both sham and injured mice at the same time point, in muscle as well as in the spinal cord at the level of SCI 24hr post-injury. P2RX3 receptor is a ligand-gated ion channel (ligand is ATP) following nociceptor activation. This receptor plays a role in peripheral pain response, and is also involved in activation of macrophages (Ford, 2012). Previous work is inconsistent with the interpretation of up or down-regulation of P2RX3 mRNA levels following injury (Chen et al., 2005; Harriott and Gold, 2009; Shinoda et al., 2007). However, this may be due in part to discrepancies between injury type, severity, and timing, which may account for why we see these changes at 7d post injury, and not at 24hr post injury or in mice that received the higher severity SCI.

We also found a significant decrease in Asic2 expression in sham mice at 7 days post injury in the muscle, but a significant increase in Asic1 at 24hr post injury in mice that received the higher severity injury, as well as at 7 days in mice that received the lower severity injury. The literature has already reported a variety of complex changes in ASIC subunit expression, similar to our observations in particular; a decrease in ASIC2a expression and an increase in ASIC1b after nerve injury (Harriott and Gold, 2009). These data suggest that ASICs may have a critical role in neuropathic pain phenotypes, at least in the uninjured population of afferents. Acid sensing ion channels (ASICs) produce multiple physiological responses in sensory neurons, to both thermal and mechanical stimuli, and provide a common mechanism that may be driving the differential responses of both populations of sensory neurons (Price et al., 2000). We observed a significant decrease in Asic3 at the level of SCI in animals that received the lower injury severity 7days post injury, implicating a possible role for ASIC3 in the sensation of mechanical or thermal changes after injury (Deval et al., 2010; Jankowski et al., 2009). Surprisingly, we did not observe any changes below the level of injury in the spinal cord itself, which we predicted we would see if the spinal cord was playing a role in the transduction of below level pain.

However, previous work has shown that primary nociceptors exhibit persistent and spontaneous activity after SCI, suggesting that perhaps below level pain is more dependent upon continual hyperexcitability of peripheral neurons in the DRG rather than in the dorsal horn of the spinal cord below the level of injury (Yang et al., 2014). Supporting this idea, DRG located below the level of injury have increased expression of P2ry1 24hrs after injury as well as an increase in Calcα, Gfrα1, Gfrα3, and Trpa1 expression 7 days after injury. We did still observe a decrease in Trpv1 at both 24hrs and 7 days in DRG, below the level of injury. Additional studies have shown that the expression of TRPV1 in neurons decreases after peripheral axonal damage (Lauria et al., 2006). Activation of both TRPV1 and TRPA1 not only transduces afferent signals to the spinal cord, but also efferent signaling via secretion of inflammatory components such as substance P and CGRP. The increase of Trpa1 in DRG below the level of injury may therefore be contributing to neurogenic inflammation after injury (Julius and Basbaum, 2001). TRPA1 can also be activated by calcium, and it is possible that TRPA1 is also functioning to amplify other signaling transduction pathways that respond to increases in intracellular calcium (Patapoutian et al., 2009). The role of P2RY1 in the DRG is less clear, but it is thought to be expressed in a subpopulation of afferents that also contain P2RX3 and lack TRPV1. P2RY1 responds to ADP and has been shown to respond to changes in heat as well as some changes in cold stimuli (Jankowski et al., 2012; Molliver et al., 2005). Changes in the expression of this receptor may also contribute to the reported below level burning sensation that patients report after SCI.

Changes in single cell gene expression

While gene expression is typically analyzed in whole tissue samples, this methodology may attenuate gene expression changes that can occur within individual neurons (Ginsberg et al., 2004). Persistent pain may be attributed to changes in the properties of peripheral nerves as a consequence of injury or disease or by inflammatory responses that produce changes within the chemical environment surrounding neurons (Basbaum et al., 2009). In sham treated mice, we

observed a significant decrease in Asic1 in cells backlabeled with WGA-488. There is not a significant decrease in Asic1 in the non-peptidergic specific subset of cells, however there is a significant decrease in Asic1 in the subset of dual-function neurons that are both peptidergic and non-peptidergic. It is possible that peptidergic cells specifically exhibit a decrease in ASIC1, which is known to contribute to central sensitization of pain (Wemmie et al., 2013). We also observed a decrease in Gfrα3 in the non-peptidergic subpopulation of cells. GFRα3 is part of the glial-cell line-derived neurotrophic factor (GDNF) family. Neurotrophic factors have been proposed to play an important role in the response of nociceptors following injury, and previous work has shown the involvement of GFRα3 in nociceptor sensitization via its ligand, artemin (Jankowski et al., 2010). However, whether artemin and GFRα3 are nociceptive or anti-nociceptive is still uncertain (Gardell et al., 2003).

Despite changes in specific gene expression in sham mice, we observed changes in a separate subset of genes in cells from SCI mice, suggesting a specific role for Trpa1 (both peptidergic and non-peptidergic cells) in both injury severity levels tested. Trpa1 expression is significantly increased 7 days post injury in whole tissue DRG below the level of injury, suggesting that this initial decrease in Trpa1 expression early after injury is transient. TRPA1 may contribute to detection of noxious cold stimuli as well as mechanotransduction (Caterina et al., 2000; Patapoutian et al., 2009; Stucky et al., 2009). In the non-peptidergic specific group of nociceptors, Asic3 expression is increased at the higher severity injury, whereas Gfrα3 expression is significantly increased with a lower injury severity. ASIC3 is expressed only in sensory neurons, has previously been proposed to modulate pain conditions associated within a variety of tissues, and is subject to modulation by precise changes in pH ranging from 6.2-6.8 (Benson et al., 2002; Lingueglia, 2007). While many of these changes do not directly corroborate with changes observed at the whole tissue level, it is likely that these genes are specific for changes below the level of injury in the hairy hindpaw skin.

Behavioral Changes

Both complete and partial spinal lesions result in the development of chronic central pain syndromes in the majority of SCI patients (Christensen and Hulsebosch, 1997; Yezierski, 1996). While nociceptive pain is associated with transient discomfort, neuropathic pain is correlated with lesions to the nervous system and is characterized by persistent pain hypersensitivity (Ji et al., 2003). This includes hyperalgesia and allodynia, as well as pain experienced in the absence of peripheral stimulus. SCI triggers persistent alterations in nociceptors that drive prolonged hypersensitivity and pain after severe injury and inflammation in the periphery. Genetic approaches to study peripheral sensory neurons have primarily focused on identifying individual molecules that conduct noxious stimuli. However, pain modalities are distinguished in large part by which neurons are activated by these specific molecules (Cavanaugh et al., 2009). Because the nociceptor population is heterogeneous, understanding behavioral function can help elucidate how these primary afferents behave after injury. Although we observed a significant difference in mechanical response threshold in injured mice that persisted from 4-28 days after SCI, this is not a surprising finding, as we did not anticipate an ability of these mice to respond to mechanical stimulation until their locomotive behavior improved. However, we were surprised to see similarities in mechanical response threshold between SCI mice and sham or naïve mice 24hrs following injury, suggesting that within the first 24hrs after injury these mice are still responsive, despite their inability to move their hind paws, and that the system is still somewhat plastic. This introduces the possibility that an acute 24hr window occurs in which the sensory system would likely still be adaptable to improvement following targeted treatment for pain. Although we did see a significant increase of Trpv1 in sham treated mice 7 days post injury, and TRPV1 activation is known to play an important role in behavioral hypersensitivity, we did not observe any significant differences in the mechanical threshold of sham mice at 7 days (Wu et al., 2013). However, we did observe a significant decrease in thermal hypersensitivity at 24hrs in sham mice. This lack of

responsiveness may in fact be due to gene expression changes that we observed in Trpv1, as TRPV1 function is known to drive reflex hypersensitivity (Yang et al., 2014).

Conclusion

Our study demonstrates that there are changes in whole tissue gene expression below the level of SCI as early as 24hrs post-injury, as well as up to 7 days post-injury. These changes in gene expression differ from those observed in sham animals, indicating that the changes observed are due to injury to the spinal cord itself and not due to the laminectomy. The results reveal several possible targets that may be involved in the generation of pain after injury. This is demonstrated further in the changes we observed at the single cell level as early as 24hrs following SCI. Although we did not observe mechanical or thermal behavioral hypersensitivity within the 28-day time frame, we did find that SCI animals do not differ in mechanical sensitivity within the first 24hrs after injury, suggesting a period of plasticity following injury despite a score of 0 on the Basso Mouse Scale (immobile). It is also important to note that the behavioral tests performed did not examine chronic pain phenotypes, and it is possible that, if we had examined the mice for a longer period of time, we would have been able to better assess chronic behavioral hypersensitivity.

Further work needs to be conducted to better determine a non-biased panel of genes that are involved post-injury, with additional consideration for timing after injury as well as injury severity. We found that that the time period following injury contributes substantially to changes in gene expression, and differs even within the same tissue type at different injury severities. By taking a non-biased approach to establish gene changes involved in injury, we can define a narrow panel of gene targets to either knockdown or inhibit before injury, as well as to work towards determining a mechanism involved in the generation of pain after SCI. Additional work also needs to be completed to study how physiological response profiles of below-level nociceptors are altered in SCI mice.

Nociceptor afferents may function as an ideal site for intervention as they contribute to hypersensitivity following injury, and are located in the periphery where they are easily accessible for treatment administration. While recent advances have been made to better elucidate how nociceptor afferents are involved in the development of SCI-induced hypersensitivity, there still remains a need to garner a better understanding of alterations within sensory afferents from the PNS following SCI. An improved understanding of the function of subsets of primary sensory neurons is fundamental to elucidate specific targets for pain treatment.

Methods

Animals

Experiments were conducted with adult (7-12 week) female C57/BL6 mice (Jackson Laboratory). Although several studies have reported higher pain prevalence in the SCI population among female patients, the majority of research has examined SCI in male rodents (Cardenas et al., 2004; Kehlet et al., 2006; Werhagen et al., 2007). Women also report greater frequency, severity, and longer lasting pain, as well as neuropathic pain below the level of injury, in comparison to men (Norrbrink Budh et al., 2003; Werhagen et al., 2007). This study will contribute to what is known in the literature by studying female mice (Cardenas et al., 2004). Naïve animals were group housed and injured animals were individually caged. All animals were maintained on a 12:12-h light-dark cycle with a temperature-controlled environment, and given food and water ad libitum.

Behavioral tests

Recovery of hindlimb motor function

Functional recovery of hindlimb movement was assessed using the Basso Mouse Scale (BMS). Mice were observed individually in an open field that met the parameters outlined in Basso et al (Basso et al., 2006). Hindlimb motor function was assessed over a 4 min. period by two investigators and scored according to the BMS standards (0= no ankle movement, 3= plantar

placement of paw, 6= plantar stepping and some coordination, 9= consistent stepping, coordination, trunk stability, normal tail placement) Mice were evaluated at days 1, 4, 7, 14, 21, and 28 post-injury (n= 8 per group).

Thermal sensitivity

To examine heat sensitivity, mice were placed in a Plexiglas box on top of a Plexiglas platform that allows for stimulation of the tail with an infrared light heat source (Hargreaves plantar test apparatus) (Hargreaves et al., 1988). Prior to testing, mice were acclimated to the apparatus for 60 minutes. Thermal reactivity was tested by the Hargreaves radiant heat method, as described (Hargreaves et al., 1988). Briefly, an infrared heat stimulus was presented to the last 0.5-1cm of the tail and withdrawal latency served as index of sensitivity. Thermal hypersensitivity was defined as a decrease in tail withdrawal latency in response to a radiant stimulus after injury. The thermal stimulus stopped when the tail moved or after 30 seconds (to prevent tissue damage). The stimulus was conducted 5 times, at 10 minute intervals, and the first 2 scores were dropped from final analysis. The tail was used instead of the hind paw in this test as it mediates a spinal reflex to nociceptive stimuli, as well as circumvents the issue of differences in hind paw placement and direction after paralysis (Gardmark et al., 1998). Mice were tested for thermal hypersensitivity 1 day prior to SCI for baseline response thresholds, and at days 1, 4, 7, 14, 21, and 28 post-injury (n= 8 per group).

Mechanical sensitivity

To assess mechanical sensitivity, mice were placed in square Plexiglas containers on a wire mesh platform. Prior to testing, mice were acclimated to the apparatus for 60 minutes. Mechanical reactivity was assessed on the plantar surface of the hind paw using a series of calibrated von Frey filaments according to the up-down method as described (Dixon, 1980), and 50% response thresholds were compared across all conditions. Both hindpaws were tested for mechanical

sensitivity, and collapsed across each group of mice per condition. Mice were tested for mechanical hypersensitivity 1 day prior to SCI for baseline response thresholds, and at days 1, 4, 7, 14, 21, and 28 post-injury (n= 8 per group).

Spinal cord injury (SCI) procedure

Animals were anesthetized by inhalation of isoflurane and a 1.0- cm dorsal midline skin incision was made over T8-T11, as per (Ma et al., 2001). Connective and muscle tissue were removed to expose the bone from T9-T10, and a laminectomy was performed at the T10 vertebral level. SCI-treated mice were placed under an impactor (Infinite Horizons IH- 0400) to receive a contusion injury (70 kDyne impact, 10 s dwell time, or 65 kDyne impact, 1 s dwell time), while sham control mice received the same treatment excluding the impact. One kilodyne is defined as a unit of force that, acting on a mass of 1kg, increases its velocity by one centimeter per second for every second along the direction it acts. Mice were allowed to recover in warm cages for 24hr. All animals were administered antibiotics once immediately following surgery (5mg/kg gentamicin), as well as saline for 4 days following surgery. Manual bladder expression was performed twice daily.

Backlabeling procedure

To backlabel the saphenous nerve, mice were anesthetized with isoflurane. A small incision was made mid-thigh to expose the saphenous nerve, as per (Christianson et al., 2006). Fine iridectomy scissors were used to separate the saphenous nerve from the blood vessel, and conjugated dyes were pressure injected into the saphenous nerve using a glass microelectrode connected to a picospritzer. WGA-488 or IB4-488 conjugated antibody were injected into the saphenous nerve to backlabel all cutaneous afferents or nonpeptidergic only, respectively. This technique does not cause significant injury to the sensory afferents being studied.

Primary DRG neuron culture

Mice were anesthetized with an intramuscular hindlimb injection of ketamine (90mg/kg) and xylazine (10mg/kg) and perfused with ice cold sterile 0.9% NaCl. L2 and L3 DRG from the side of nerve injection were dissected into cold HBSS, after which the mice were sacrificed by decapitation. Culture was performed as described (Malin et al., 2007). Briefly, following collection, tissue was treated with 60U papain (Worthington), 1mg of cysteine, and 6µL of $NaHCO_3$ in 1.5mL HBSS at 37°C for 10 min. Tissue was then treated with 12mg collagenase II (Worthington) and 14mg dispase (Roche) in 3mL HBSS at 37°C for 20 min., washed, and triturated with fire polished glass Pasteur pipettes in 1mL of DMEM supplemented with FBS and pen/strep. Cells were plated on poly-D-lysine coated coverslips and allowed to sit for 2 hours prior to single cell pickup.

Single cell pickup

Following primary DRG cell culture, coverslips were transferred to single cell solution (140 mM NaCl, 10 mM Glucose, 5 mM KCl, 10 mM Hepes, 2mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4) (Citri et al., 2011). Fluorescently labeled cells were identified for pickup by bright field illumination. Using a large bore glass electrode and a micromanipulator connected to a syringe, fluorescent cells that were not surrounded by debris or attached to neighboring cells were individually picked up and transferred directly into 3µL QuickExtract™ RNA Solution (Lucigen), briefly centrifuged, snap-frozen on dry ice, and stored for subsequent use (n=4 mice/condition, 20-40 cells/gene target).

Tissue collection

Mice were anesthetized with an intramuscular hindlimb injection of ketamine (90mg/kg) and xylazine (10mg/kg) and perfused with ice cold sterile 0.9% NaCl. Hairy hindpaw skin and quadriceps muscle were collected from both sides of the animal, snap-frozen in liquid nitrogen, and stored at -80°C for subsequent use. Spinal cord and DRG tissues were dissected at (T9-T11),

and below (L1-L3) the level of injury, snap-frozen on dry ice, and stored at -80°C for subsequent use (n=4-7/condition).

RNA extraction and PCR

RNA isolation was performed using the suppliers' protocol from Qiagen RNeasy mini kit, fibrous tissue kit, or micro kit, for hindpaw hairy skin and spinal cord, quadriceps muscle, or DRG, respectively. RNA isolation from single cells and reverse transcription was performed using the suppliers' protocol from Message*BOOSTER*™ cDNA synthesis from Cell Lysates Kit (Lucigen). 500ng of total RNA was reverse transcribed using Iscript cDNA kit (BioRad) with a mix of oligo(dT) and random primers and stored in -20°C until used for real time PCR (RT-PCR) reactions. cDNA was added to SYBR Green MasterMix (BioRad) and run in duplicate on an Applied Biosystems Imager (program 95 °C, 30 sec; 95 °C, 15 sec; 60 °C, 60 sec; repeat 40x). RT-PCR reactions were similar to whole tissue, differing only in regards to less stringent cDNA values. Cells were eliminated from analysis if the Gapdh Ct values were above 26. Values were first normalized to Gapdh, and then to average sham or naïve control values. Fold change is reported as $2^{\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Primer sequences

Table 2-1 Primers used in Real-time RT-PCR

Target	Forward Sequence	Reverse Sequence
Asic1	CAGGCCAGCTCTCCAATCTC	ACGTACACAGGTGATCTGCC
Asic2	GCACCTGTGGAGGAAGTACG	CCCGCCCCAAACAAAAATCAG
Asic3	GCAACACTCTGCTCCAGGAA	CGAGGTAACAGGTACGGTGG
Calcα	TGACAGCATGGTTCTGGCTT	GTCCCCAGAAGAGCAAGAGG
Gapdh	ATGAATACGGCTACAGCAACAGG	CTCTTGCTCAGTGTCCTTGCTG
Gfrα1	GACTCGGAATCCAGCCTACG	TCTGCACTTGTCCTCTCGTG
Gfrα2	CTTGGGGAGAAGGGCTGTTG	AGGAGAAGAGAGAAGGGGCA
Gfrα3	GCTGGTGTCTTGACTGCTCT	GACCCAAGGGACTAGGGGAA
Mrgprd	CTGCTTCAGGCCAGCTCCTA	AGCATCTCTGTCACCTTGAGCA
P2rx3	GGTGCCTAAGCCTCTTCTGG	AGGGATGGCGCTGAGTAAAC
P2ry1	GCCAGGACACTAACCCATCG	AACTGAAGGCCCACAAACCTC
Trka	GAGCCAAGTTTTGGTGCCAG	GATGCTGGCCATGAAGCAAG
Trkb	AAGATGTGTCCCTGGGCTTC	AAGTGAGTCATGAGCTGCC
Trkc	CTGCTCCCCATGGTTGTAGG	GGGAGGCTGGAAATGAGGTC
Trpa1	TGAGCCACATGACAGAAGTCC	CTAAGCAGCAGCAACAACTGG
Trpv1	GGCGAGACTGTCAACAAGATTGC	TCATCCACCCTGAAGCACCAC

Statistical analysis

A two-way ANOVA followed by a Tukey's test was used to make whole tissue comparisons as well as for behavioral tests where there were more than two conditions. We used an unpaired Student's t-test to compare individual single cell gene transcript changes with either sham or naïve conditions. All statistics on PCR data were conducted using delta CT values and are graphed as fold change. Prism software (GraphPad) was utilized for all statistical tests.

Samples for the experiments described in Chapter 2 were collected and analyzed in the laboratory of Assistant Professor Kyle Baumbauer. Associated genetic analyses were performed in the laboratory of Assistant Professor Erin Young.

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CHAPTER 3

Transcriptional profiling of non-injured nociceptors after spinal cord injury reveals diverse molecular changes

This chapter was submitted to *Scientific Reports*. JRY and REM designed research; JRY performed research; JRY and REM analyzed data; JRY, ILM and REM wrote the paper.

Abstract

Traumatic spinal cord injury (SCI) has devastating implications for patients, including a high predisposition for developing chronic pain distal to the site of injury. Chronic pain develops weeks to months after injury, consequently patients are treated after irreversible changes have occurred. Nociceptors are central to chronic pain; the diversity of this cellular population presents challenges to understanding mechanisms and attributing pain modalities to specific cell types. Experimentally, thoracic SCI was induced in mice using a vessel clip. A major cause of increased nociceptor activity after SCI could be alterations in gene expression, therefore SCI-induced transcripts were examined in dorsal root ganglia (DRG) distal to the site of injury to determine if alterations in gene expression were associated with increased nociceptor activity after SCI. Sensory neurons projecting to the hairy hindpaw skin were retrograde labeled with fluorescent dye. Lumbar DRG below the injury were collected post-injury, dissociated, and labeled neurons were purified by fluorescence-activated cell sorting. RNA was extracted from sorted sensory neurons of naïve, sham, or SCI mice and sequenced. Transcript abundances validated that the desired population of nociceptors were isolated. Differential gene expression analysis showed significant transcript changes in SCI cell populations relevant to the onset of chronic pain.

Introduction

While spinal cord injury (SCI) is typically associated with loss of locomotor function, it can also result in chronic pain, affecting nearly 70% of patients with SCI (Finnerup et al., 2001). There are many categories of pain types affecting this population however, studies indicate that neuropathic pain below, or distal, to the level of injury is among the most common and difficult to treat (Defrin et al., 2001; Finnerup et al., 2001; Nees et al., 2016; Siddall and Loeser, 2001; Yezierski, 2005). Of those patients reporting below-level neuropathic pain, half described their pain as severe or excruciating, causing significant disability in patients already disabled from loss of motor function (Defrin et al., 2001; Siddall et al., 2003). With few patients able to achieve complete relief with current treatment options, research has focused on mechanisms responsible for SCI pain at the site of injury, with the intention of treating the injury itself to prevent subsequent development of pain.

Considerable advances have been made in understanding changes within the spinal cord, including how spinally mediated alterations contribute to SCI-induced pain by increasing spinal cord excitability, and by establishing a variety of factors that impact how incoming sensory stimulation is processed (Bruce et al., 2002; Meisner et al., 2010; You et al., 2008). However, this approach has not translated into successful pain management. This may be attributed to an incomplete understanding of the differential functions of specific afferent subtypes in SCI, and how afferents distal to the site of injury become sensitized in patients with chronic below-level pain (Thakur et al., 2014). The sensory system receives inputs from multiple cell types, and peripheral cell bodies within the dorsal root ganglion (DRG) are important targets for assessing sensory function and pain (Usoskin et al., 2015). Persistent activity from injured and non-injured afferent fibers contributes to development and maintenance of chronic pain following SCI (Gold and Gebhart, 2010).

Each sensory neuron has a unique pattern of gene expression that influences its modality-specific contribution to injury-induced pain. To better understand the underlying pathophysiology of below-level pain following SCI, it is necessary to identify changes in cells impacted by the injury. The skin is heavily innervated by a broad range of nociceptors, and previous work has shown that SCI can impact the function of cutaneous nociceptors below the level of injury (Berta et al., 2017). This has been demonstrated by sustained spontaneous activity in peripheral terminals and in cell bodies of sensory neurons projecting to the skin after initial SCI (Carlton et al., 2009; Defrin et al., 2001). Additional work has demonstrated that blockade of peripheral afferents into the central nervous system can effectively mitigate patient discomfort and chronic pain. These data support the idea that the mechanisms generating and maintaining prolonged pain reside within the peripheral nervous system (Basbaum et al., 2009; Campbell et al., 1988; Gold and Gebhart, 2010).

In the present study, we identify specific transcriptional alterations in non-injured DRG distal to the site of injury. Using retrograde labeling from hairy hindpaw skin and flow cytometry, we isolated a nociceptor population projecting to sites distal to the spinal injury, free of surrounding neuronal and glial cells. This enabled identification of novel cutaneous nociceptor genes and predicted pathways not discernible by whole DRG tissue analyses.

Results

Characterization of behavioral and inflammatory phenotypes of sham and injured mice

To determine an optimal time point to observe transcriptional changes contributing to the transition from acute to chronic pain, we tested behavioral differences between naïve and sham operated mice. This was to ensure that changes within the DRG were due to injury to the spinal cord itself, not to the laminectomy performed in both injured and sham mice. Specifically, we tested naïve and sham mice for open field locomotor differences 1, 3, 5, and 7 days post-surgery

(**Fig. 3-1A1**). Naïve and sham mice did not differ significantly at any of the time points tested, including as early as 1 day post-surgery. To ensure that SCI mice did exhibit behavioral differences and locomotor deficits following injury, we compared SCI mice to naïve and sham mice for open field behavior 1 day post-injury (**Fig. 3-1A2**). SCI mice exhibited substantially decreased total ambulation (one-way ANOVA, $p=0.0059$, Tukey's multiple comparisons test, naïve $^{**}p<0.005$, sham $^{*}p<0.05$) following injury, as expected, although time spent in the periphery did not differ (**Suppl. 3-1A**). Additional tests for mechanical (von Frey) and thermal (hot water tail-flick) hypersensitivity showed that naïve and sham conditions did not differ significantly at any time point (**Fig. 3-1B**). We also examined post-surgery changes in inflammation, using ELISAs to analyze inflammatory cytokine levels (TNF-α, IL-6, IL-1β, IL-10) in extracts of spinal cord segments (T8-T11) in naïve or sham mice at 1, 3, 5, and 7 days post-surgery (**Fig. 3-1C**, **Suppl. 3-1B**). Spinal cords from SCI mice had significantly increased levels of IL-6 and IL-10 in comparison to naïve controls (one-way ANOVA, Bonferroni's multiple comparisons test; $^{**}p<0.005$, $^{**}p<0.005$, respectively), however we did not observe significant changes in inflammatory cytokine levels in sham mice. As a positive control, we tested cuprizone-treated mice, which are known to secrete proinflammatory cytokines at the end of 5 weeks of treatment. Cuprizone-treated mice showed expected increases in levels of TNF-α, IL-1β, and IL-10 in comparison to naïve controls (one-way ANOVA, Bonferroni's multiple comparisons test; $^{**}p<0.005$, $^{**}p<0.005$, $^{***}p<0.001$, respectively) (Schmitz and Chew, 2008). Behavioral testing and cytokine analyses did not reveal differences between naïve and sham mice at any time point.

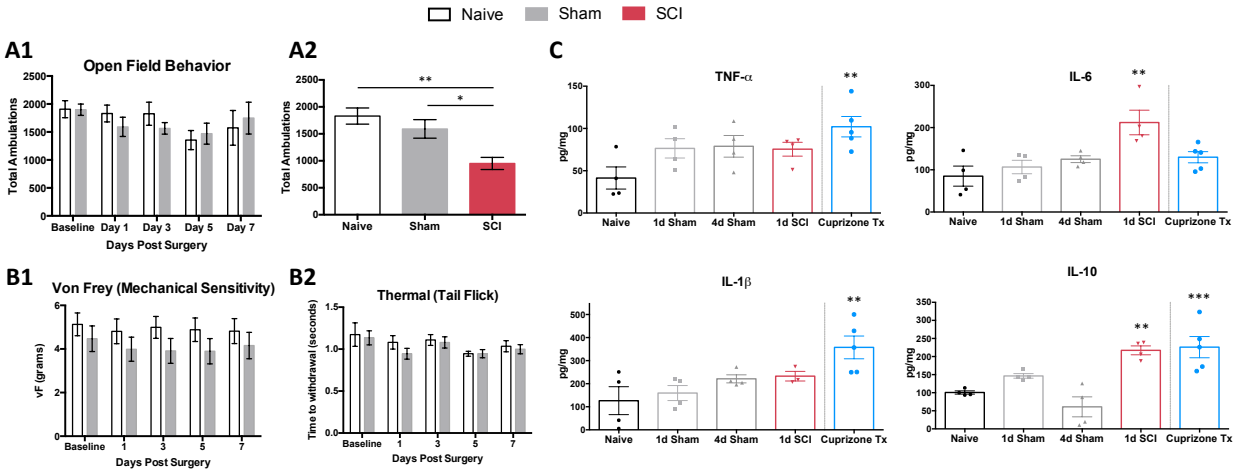

Figure 3-1. Behavioral responses and cytokines. (A1) Open field behavior (10 minute trials) in naïve or sham mice 0,1,3,5 and 7 days post-surgery does not differ significantly at any time point in total ambulation, N=3-10 sham, 4 naïve. (A2) Total ambulation differs significantly 1 day post-SCI in both SCI vs. naïve (one-way ANOVA, p=0.0059, Tukey's multiple comparisons test **p<0.005) and SCI vs. sham (*p<0.05) mice. (B1) Mechanical and (B2) thermal sensitivity do not differ significantly 0,1,3,5, and 7 days post-surgery in naïve and sham mice, N=6 each. (C) Cytokine ELISAs on spinal cord segments at the level of laminectomy (T8-T11) show no significant differences between naïve and sham mice 1 or 4 days post-surgery. 1-day post-operation, SCI mice have significantly increased levels of IL-6 and IL-10 compared to naïve controls (one-way ANOVA, Bonferroni's multiple comparisons test; **p<0.005, **p<0.005, respectively). Cuprizone treated mice as positive control, with significantly increased TNF-α, IL-1β, and IL-10 compared to naïve controls (one-way ANOVA, Bonferroni's multiple comparisons test; **p<0.005, **p<0.005, ***p<0.001, respectively) after 5 weeks of cuprizone treatment.

Confirmation of cell population specific labeling of cutaneous nociceptors

Because the laminectomy did not significantly affect behavior or inflammatory responses in sham mice, we determined an optimal time point to study the transition of acute to chronic pain based upon established characteristics of nociceptors following SCI. Previous studies have confirmed onset of spontaneous activity in nociceptors distal to the site of SCI as early as 3 days post-injury; increased activity persisted for at least 8 months (Bedi et al., 2010). Thus, it is possible that the transition to chronic pain begins around 3 days after SCI, and this transition is not due to laminectomy as nociceptors isolated from sham mice show no significant changes in spontaneous activity (Bedi et al., 2010; Yang et al., 2014). Subsequently, we chose 4 days post-injury to assess transcriptional changes in nociceptors below the level of injury.

To perform transcriptional profiling on nociceptors that project to the cutaneous skin after injury, we injected wheat germ agglutinin conjugated to an AF-488 dye (WGA-488) into the sural, common peroneal, and saphenous nerve skin territories for retrograde labeling of DRG neurons, performed SCI or sham surgeries 2 days post-WGA injection, and collected L2-L6 DRG for cell culture 4 days post-injury (**Fig. 3-2A**). We used flow cytometry to confirm that our cell population of interest (cutaneous nociceptors) was positively labeled with WGA-488 (**Fig. 3-2B**). We also observed non-labeled (WGA-488-) cells and dead cells (propidium iodide, PI+), to be excluded from cell sorting and analysis (**Fig. 3-2B**).

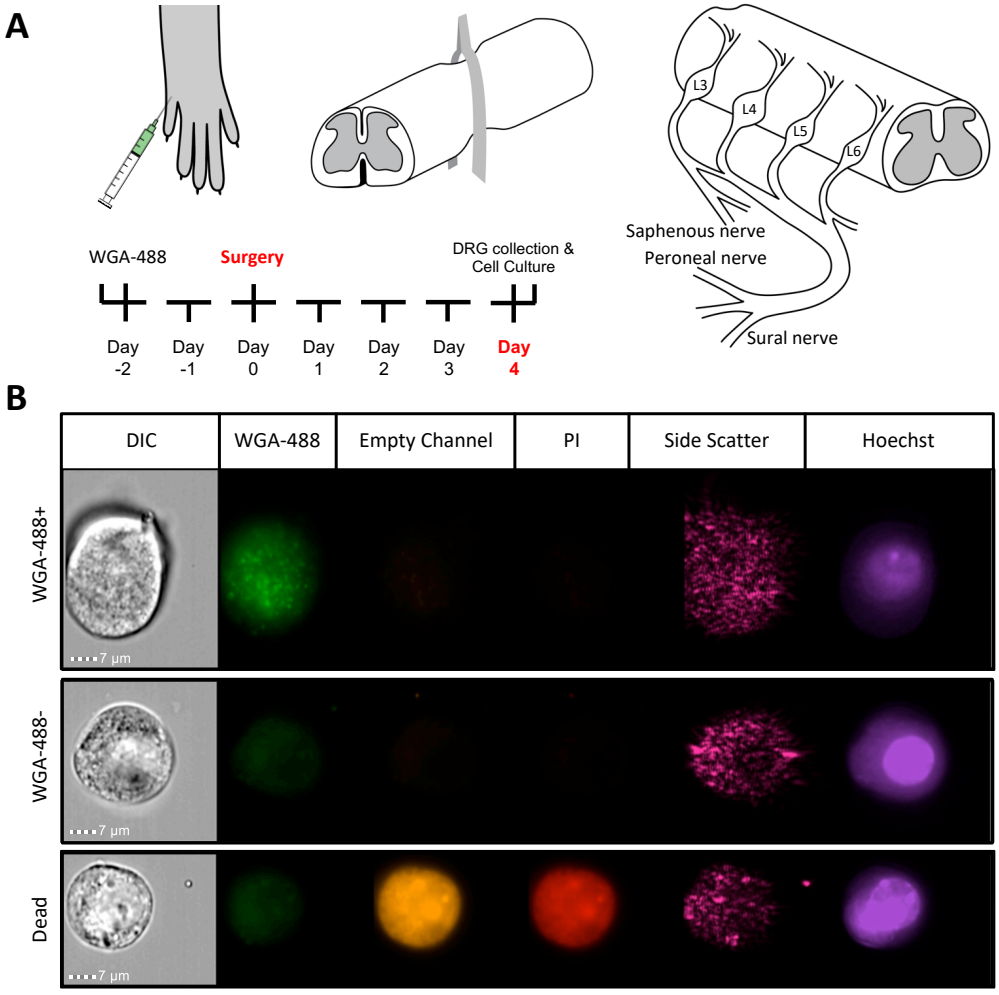

Figure 3-2. Identifying sensory neurons. (A) Diagram of WGA-488 injection under the hairy hindpaw skin of a mouse, how the spinal cord injury was conducted with a vessel clip, and projections to the corresponding DRG. (B) Fluorescent cell images taken by Amnis ImageStreamX Mark II imaging flow cytometer (Luminex Co.) using a 60x objective lens with excitation from a 405nm laser and a 488nm laser to identify propidium iodide (PI; red) positive (dead) cells and WGA (green) labeled cells, respectively.

FACS purification of DRG nociceptors projecting to the cutaneous hind paw

We performed fluorescence-activated cells sorting (FACS) purification of nociceptor populations from naïve, sham, and SCI adult (8-12 week old) female mice (n=5 per condition). We pooled DRGs from lumbar regions L2-L6 on either side of the spinal column to ensure all DRGs isolated would have projections to the hairy hind paw skin. DRG cells were enzymatically dissociated and subjected to flow cytometry, to gently isolate positively labeled cells between 10 and 30μm; propidium iodide staining was used to exclude dead cells. All conditions were gated on DRG from naïve mice that did not receive WGA-488 injections, this enabled purification of positively labeled cells (**Fig. 3-3A**). Analysis of our flow cytometry data shows that we were successful in retrogradely labeling DRG neurons projecting to hairy hind paw skin. Many positively labeled neurons were part of cell aggregates, limiting retrieval of the cell population of interest to ~2% of all dissociated cells per animal (**Fig. 3-3B**). DRG populations were sorted directly into lysis buffer and placed on dry ice to preserve transcriptional profiles at the time of isolation. RNA quality was tested using the Agilent TapeStation; a representative image of an RNA sample following FACS purification is shown (**Fig. 3-3C**).

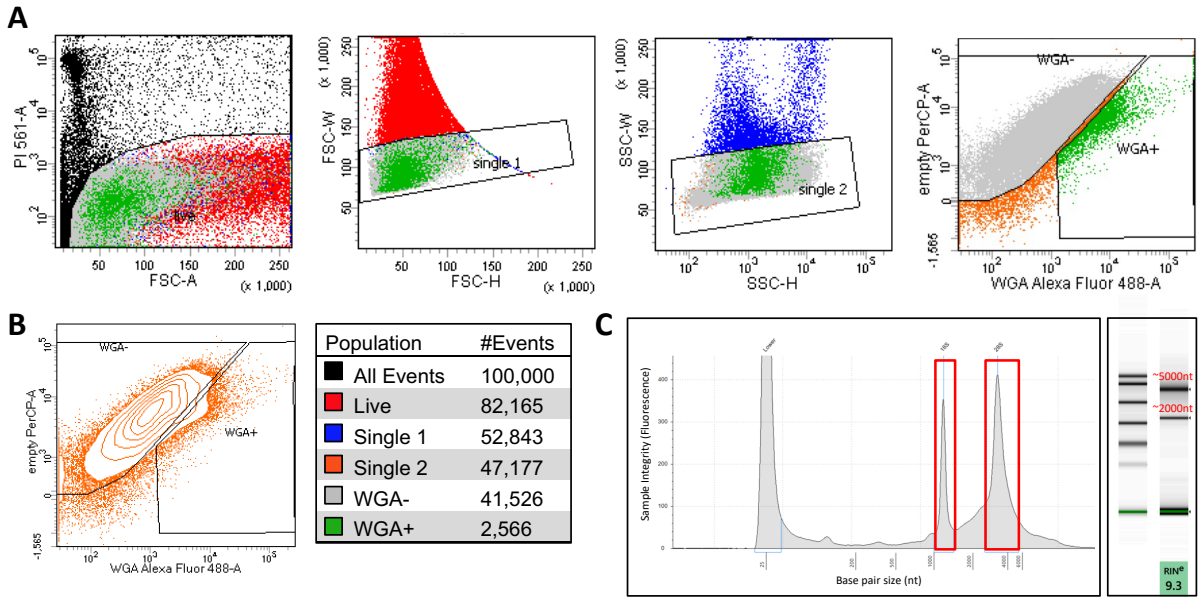

Figure 3-3. Isolating sensory neurons. (A) FACS purification of cultured DRG cells based on propidium iodide (PI) staining (dead) single cell populations by forward scatter (FSC), single cell populations by side scatter (SSC), and cells fluorescently labeled with WGA-488 (WGA+). (B) Contour plot of single WGA+ cells that were sorted directly into lysis buffer for RNA extraction and color scheme for the gated populations, each condition N=5; ~3000 cells. (C) Representative image of RIN values run on an Agilent TapeStation before RNAseq.

Major characteristics of somatosensory mediators in purified neuron population

We used the RNA sequencing data to evaluate the neuronal population that had been isolated. Scn10a, which encodes $Na_v1.8$, is present in 80-90% of nociceptors and Trpv1 serves as a marker for the peptidergic population of nociceptors (Basbaum et al., 2009; Harriott and Gold, 2009; Wu et al., 2013). RPKM values of 1000 for Scn10a and 400 for Trpv1 confirm that FACS purified cells express high levels of these nociceptor markers (**Fig. 3-4A).** The low RPKM values observed for Parvalbumin, a glial transcript also found in large diameter proprioceptors and Aβ neurons, and Gfap (glial fibrillary acidic protein), another marker for glial cells, confirmed the absence of the non-nociceptive sensory neurons responsible for touch and proprioception (Aβ and Aδ neurons) and the absence of satellite glial cells from our purified nociceptor population (**Fig. 3-4B**) (Huang et al., 2013; Le Pichon and Chesler, 2014).

We next analyzed gene expression patterns for known functional mediators of somatosensation (Chiu et al., 2014; Le Pichon and Chesler, 2014; Usoskin et al., 2015). The purified cutaneous nociceptors displayed high expression levels of genes involved in thermosensation and nociception, such as specific Trp channels (notably Trpv1), sodium channels (Scn9a, 10a, 11a) and Prph (peripherin) (**Fig. 3-4C**). Markers for non-peptidergic nociceptors were abundant, such as Mrgprd (Mas-Related G-Protein Coupled Receptor Member D), Runx1 (Runt related transcription factor 1), Ret (Ret proto-oncogene), and Trpc3 (transient receptor potential cation channel C3). As expected, transcripts enriched in peptidergic nociceptors were present, such as Calca and Calcb (Calcitonin Related Polypeptides) and Tac1 (the tachykinin precursor for peptides such as Substance P), the peptide processing enzyme PAM (peptidylglycine α-amidating monooxygenase), as well as Npy1r, one of the most abundant Npy receptors (Basbaum et al., 2009; Julius and Basbaum, 2001). However, genes encoding proteins involved in itch, such as Nppb (brain natriuretic peptide) and Hrh1 (histamine receptor H1) were expressed at low levels (Usoskin et al., 2015). Similarly, genes responsible for proteins involved in tactile function,

including Trpc1 (Transient Receptor Potential Cation Channel Subfamily C Member 1), and those responsible for proprioception, such as Runx3 (Runt related transcription factor 3), exhibited low expression levels. In contrast, nerve growth factor receptors (Neurotrophic Receptor Tyrosine Kinases 1-3 [Ntrk]) were all expressed at high levels. High expression levels were also observed for transcripts of the two major subpopulations of nociceptors; peptidergic and non-peptidergic (**Fig. 3-4D**).

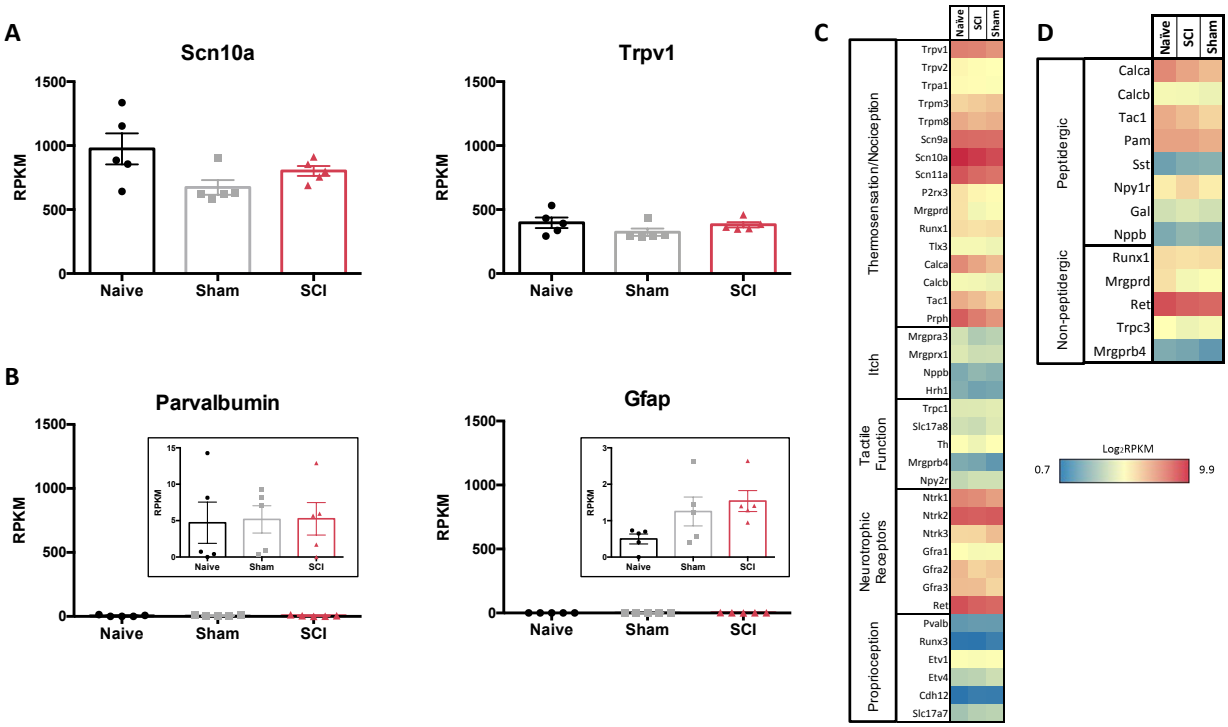

Figure 3-4. RNAseq demonstrates specificity of cell isolation. (A) Average RPKM values from RNAseq data set from genes that are known to be highly expressed in the cell population of interest or (B) expected to be expressed in other cell populations outside of the one being studied; larger myelinated neurons or satellite glial cells, respectively, N=5 per condition. (C) Heatmap of functional somatosensory mediators within the isolated cell population of interest. Genes were grouped based on roles established in the literature: thermosensation and nociception, itch, tactile function, and neurotrophic factors. (D) Heatmap of peptidergic and non-peptidergic markers. Graph: $\log_2$ of average RPKM values within each condition, RPKM >1, N=5 per condition.

Gene expression profiling and enrichment patterns in injured and non-injured cutaneous nociceptors after SCI

To further assess expression profiles of the purified nociceptor population and differences among naïve, sham, and SCI conditions within this nociceptor-enriched population, we focused on expression patterns of gene families that mediate general neuronal functions. We used differential expression analysis (DESeq2) to analyze significant changes between SCI and naïve or SCI and sham populations, requiring significant differences between SCI and naïve or sham conditions by DESeq2 and excluding outliers (Love et al., 2014). Pairwise comparisons of significant genes generated by DESeq2 analysis yielded many differentially expressed genes in each subset (**Suppl. 3-2A-B**). We also assessed differences between sham and naïve populations to exclude significant transcript changes due to changes produced by laminectomy (**Suppl. 3-2C**).

We focused on expression patterns of gene families which mediate neuronal functions and contribute to pain phenotypes, and found both high expression levels and significant differences within the chloride channel family, Trp channels, glutamate receptors, GABA receptors, potassium channels, sodium channels, and piezo channels (**Fig. 3-5A-G**, p-values in **Table 3-1**). We also examined ASICs (acid-sensing ion channels), calcium channels, glycine receptors, and P2rx and P2ry families (purinergic receptors), because these are widely studied families known to be involved in the development or maintenance of chronic pain (**Suppl. 3-3A-E**). Many channels and receptors were highly expressed within this cell population, but no significant changes among the transcripts were demonstrated for any condition (**Suppl. 3-3)**.

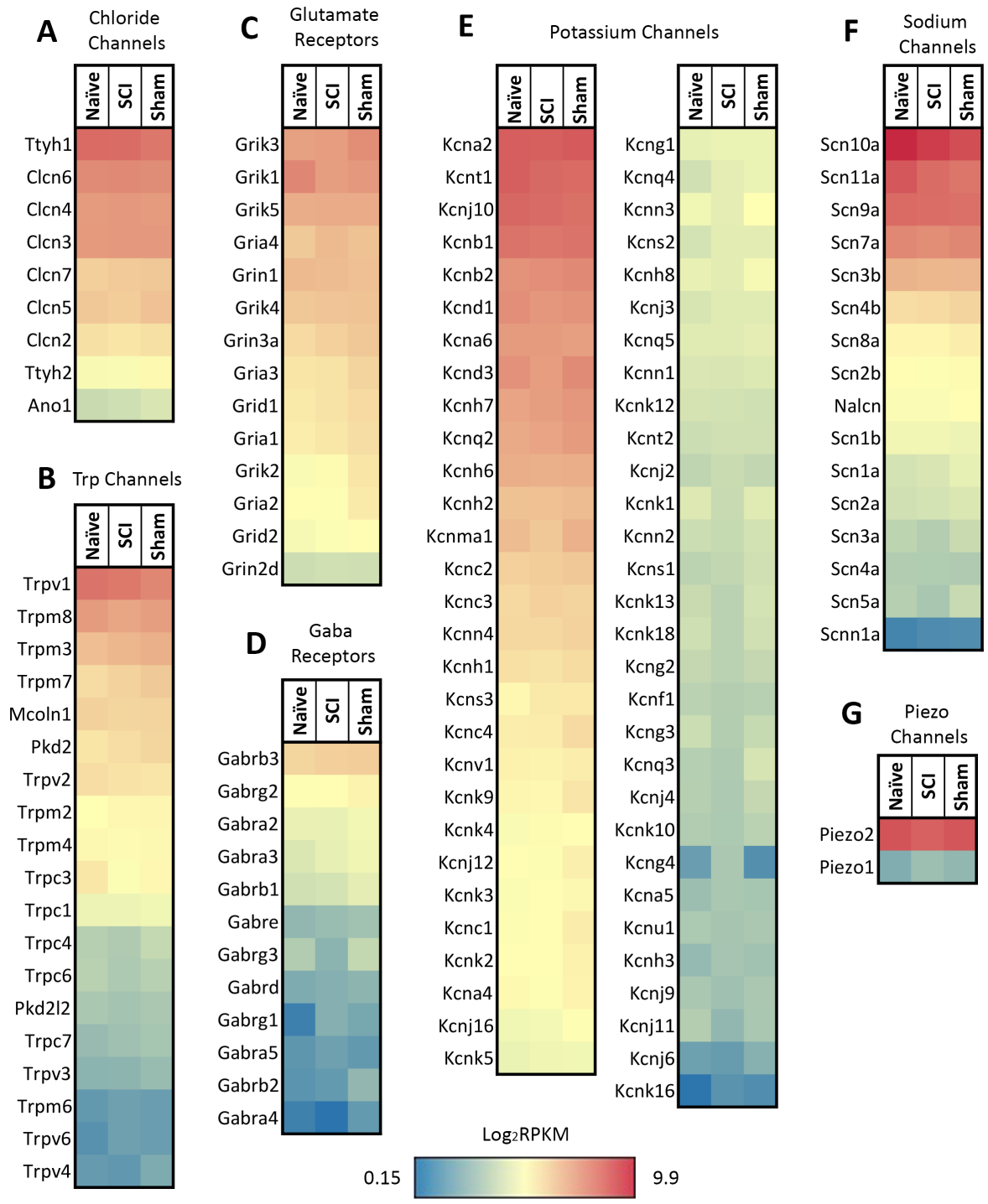

Figure 3-5. Heatmaps of ion channel transcripts. (A) Chloride channels, (B) TRP channels, (C) glutamate ionotropic receptors, (D) GABA ionotropic receptors, (E) potassium channels, (F) sodium channels, (G) piezo channels. Each family of transcripts includes significant differences in at least one gene 4 days post-SCI. Channels were clustered based on SCI expression level, and graphed by the $\log_2$ of average RPKM values within each condition. RPKM <1 were not included, N=5 per condition.

Gene	RPKM Naïve	RPKM SCI	RPKM Sham	p-Value SCI vs. N	p-Value SCI vs. Sham
Ion Channels					
Calcb	86	83	71	-	6.5E-03
Gabrg3	12	6	17	5.3E-04	5.7E-04
Gfra2	219	166	186	1.3E-03	-
Gria4	108	131	119	5.0E-02	4.5E-02
Grik1	270	196	213	4.1E-05	-
Kcng3	20	13	18	3.3E-03	1.3E-02
Kcnh8	20	13	18	-	1.2E-02
Kcnj11	34	30	44	8.3E-04	-
Kcnk1	13	7	11	2.0E-02	1.7E-02
Kcnk13	28	19	30	-	2.2E-03
Kcnk18	19	15	23	3.2E-02	1.6E-03
Kcnn3	21	15	22	-	8.8E-03
Mrgprd	40	32	51	3.7E-07	-
Piezo2	143	82	96	1.5E-02	-
Scn3a	542	447	526	2.6E-02	1.0E-05
Scn5a	27	22	34	-	8.8E-04
Trpc3	23	17	34	5.2E-04	-
Trpc4	104	74	86	-	2.3E-03
Ttyh1	18	16	25	-	2.0E-02
Synaptogenesis Pathway					
Adcy1	56	45	83	-	6.9E-05
Adcy2	120	144	140	2.9E-02	-
Ap2a2	307	305	260	-	1.6E-02
Atf4	154	147	121	-	1.3E-02
Bdnf	57	77	60	1.2E-02	3.6E-06
Cadm1	352	304	269	7.7E-02	4.5E-03
Camk2g	322	339	278	-	2.3E-02
Cdh10	35	34	46	-	9.0E-03
Dlg4	228	218	200	-	2.2E-02
Epha10	9	10	13	-	3.3E-02
Ephb2	11	9	15	-	8.4E-04
Gosr2	102	115	103	-	1.0E-02
Gria4	108	131	119	5.0E-02	4.5E-02
Nap1l1	210	203	177	-	3.1E-02
Nlgn2	202	205	185	-	9.4E-04
Plcg2	8	6	12	-	4.7E-04
Prkag2	161	169	147	-	5.7E-03
Prkar2b	182	153	147	1.5E-02	-
Rap2b	11	19	20	4.0E-03	-
Rasgrp1	364	286	303	8.1E-03	-
Rras	68	67	57	-	1.6E-02
Rras2	19	26	26	3.1E-02	-
Stxbp2	48	60	50	3.8E-02	3.0E-02
Syn3	28	27	37	-	4.0E-03
Syt4	235	231	197	-	1.1E-02

Table 3-1. Ion channels and Synaptogenesis. Average RPKM values that significantly differ 4 days post-SCI. DESeq2 p-Value based on SCI vs. naïve or SCI vs. sham comparisons. P-Values that are not listed were >0.05. Transcripts from IPA canonical pathway analysis significantly different 4 days post-SCI. DESeq2 p-Value based on SCI vs. Naïve or SCI vs. Sham comparisons.

Several transcription factors were highly expressed, including Stat3 (signal transducer and activator of transcription 3), Fos (FJB osteosarcoma oncogene), and Jun (Jun Proto-Oncogene) (**Suppl. 3-4**). Evidence from various models of neuropathic pain implicates these transcription factors in the development or maintenance of chronic pain (Dominguez et al., 2008; Harris, 1998; Naranjo et al., 1991; Tsuda et al., 2011; Xue et al., 2014). Stat3 inhibitors are used to treat peripheral nerve injury-induced hyperexcitability within dorsal horn neurons, pain behaviors, chronic constriction injury, and signaling of IL-6 cytokines (Dominguez et al., 2008; Tsuda et al., 2011; Xue et al., 2014). Previous studies also show that Fos links extracellular events to long-term intracellular changes (such as noxious stimuli) and have established Fos expression as a valid tool to study nociceptive changes (Harris, 1998; Naranjo et al., 1991). Jun also contributes to persistent pain phenotypes following injury (Naranjo et al., 1991). DESeq2 analysis determined additional transcription factors to be significantly altered by SCI (**Suppl. 3-5**).

Ingenuity pathway analysis (IPA) identified significantly different canonical pathways from cutaneous nociceptors after SCI

Based on DESeq2 analysis, levels of several hundred transcripts in the nociceptor-enriched population were altered by SCI. The IPA input lists were limited to genes that had RPKM values greater than 10 and were statistically different (SCI vs. Naïve or SCI vs. Sham) by DESeq2 analysis (**Fig. 3-6A,B**). Transcripts that exhibited large-fold changes included Mrgprb5, Hal, Chrnb4, Cap2, Sez6l, Calb1, Prokr2, Rxfp1, Nxpe2, and Arap3 (**Fig. 3-6A,B**). These genes did not appear in any common significant canonical pathways. IPA identified several pathways that are considered important for inflammatory processes, pain transduction, or the maintenance of chronic pain (**Fig. 3-6C**). This includes calcium signaling, Cxcr4 signaling, neuropathic pain signaling in dorsal horn neurons, opioid signaling, purinergic receptor signaling and synaptic long term potentiation **(Fig. 3-6C)** (Julius and Basbaum, 2001; Walters, 2012, 2018). This study

focused on significant changes due to SCI pain and not due to post-surgical pain (i.e. changes between sham vs. naïve groups).

Validation of RNASeq data using qPCR

We used qPCR to confirm changes in transcripts of interest from our RNAseq data set. To validate qPCR and RNAseq comparisons, we compared qPCR or RPKM Log_2 transcript levels of SCI and sham genes of interest (**Fig. 3-6D**). SCI and sham qPCR fold change results were analogous to the RNAseq fold change data set. We focused on the synaptogenesis pathway in particular, as it includes several genes present in overlapping canonical pathways, including changes in receptors involved in organization of excitatory signaling (Ephb) and synapses which may be involved in development of chronic pain (TrkB and BDNF). We validated receptors for significant genes in the synaptogenesis pathway, and genes considered possible contributors to pain that also showed significant differences between conditions (Gabrg3, Il6st, Kcng3, Piezo2, Scn5a, Trpc3) (De Jongh et al., 2003; Deng et al., 2018; Devor, 2006; Eijkelkamp et al., 2013; Guptarak et al., 2013; Szczot et al., 2018; Waxman et al., 1999; Wickenden, 2002; Xia et al., 2015). Additional targets were chosen in order to validate isolation of the correct cell population (Scn10a). Samples for qPCR were collected by backlabeling cutaneous afferents and cell sorting, consistent with samples generated for RNAseq analysis. Following cDNA synthesis, samples were subjected to gene target-specific preamplification using the same primers used for qPCR (**Suppl. 3-6**). Hprt was chosen as the housekeeping gene for qPCR analysis because it was the most constant normalizer transcript from the RPKM data across all 15 mice in the present study (Klenke et al., 2016; Lima et al., 2016).

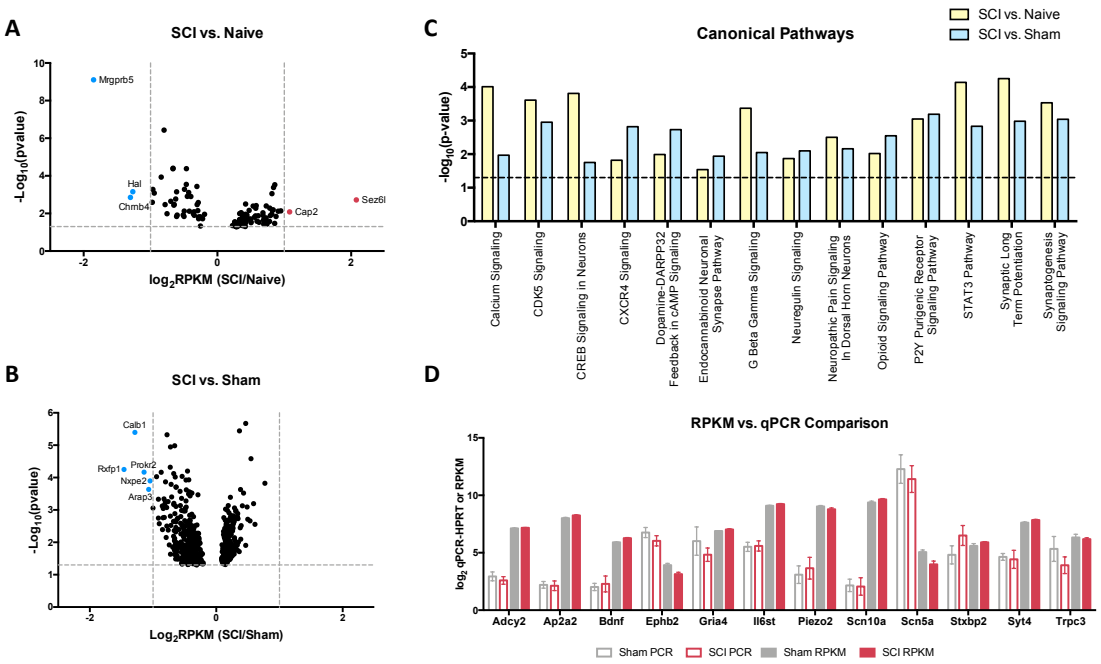

Figure 3-6. Volcano plots. (A,B) DESeq2 significant (p>0.05) genes included in IPA analysis comparing SCI vs. naïve or SCI vs. sham conditions. (C) Significant (p<0.05) overlapping canonical pathways predicted by analyzing genes determined by DESeq2 and outlier cutoff in IPA. (D) Comparisons of RPKM values generated by RNAseq and log2 transcript levels (gene-Hprt) validated by qPCR between sham and injured conditions.

IPA network analysis revealed several regulatory interrelationships after SCI

We used IPA upstream network analysis to further interpret the function of the several hundred transcripts significantly altered determined by DESeq2. This method predicted several transcriptional regulators associated with altered expression levels of downstream target genes following SCI (**Fig. 3-7A**). For each of the networks shown, the upstream regulator molecules at the center of the diagram do not show a significant change in RNA expression in response to injury. However, these targets are activated by posttranslational modifications that can alter many of the downstream molecules within its network (**Fig. 3-7B,C**). All of the relationships and log_2 RPKM values for neuronally relevant transcripts were determined to be significantly altered by DESeq2 (**Fig. 3-7D**).

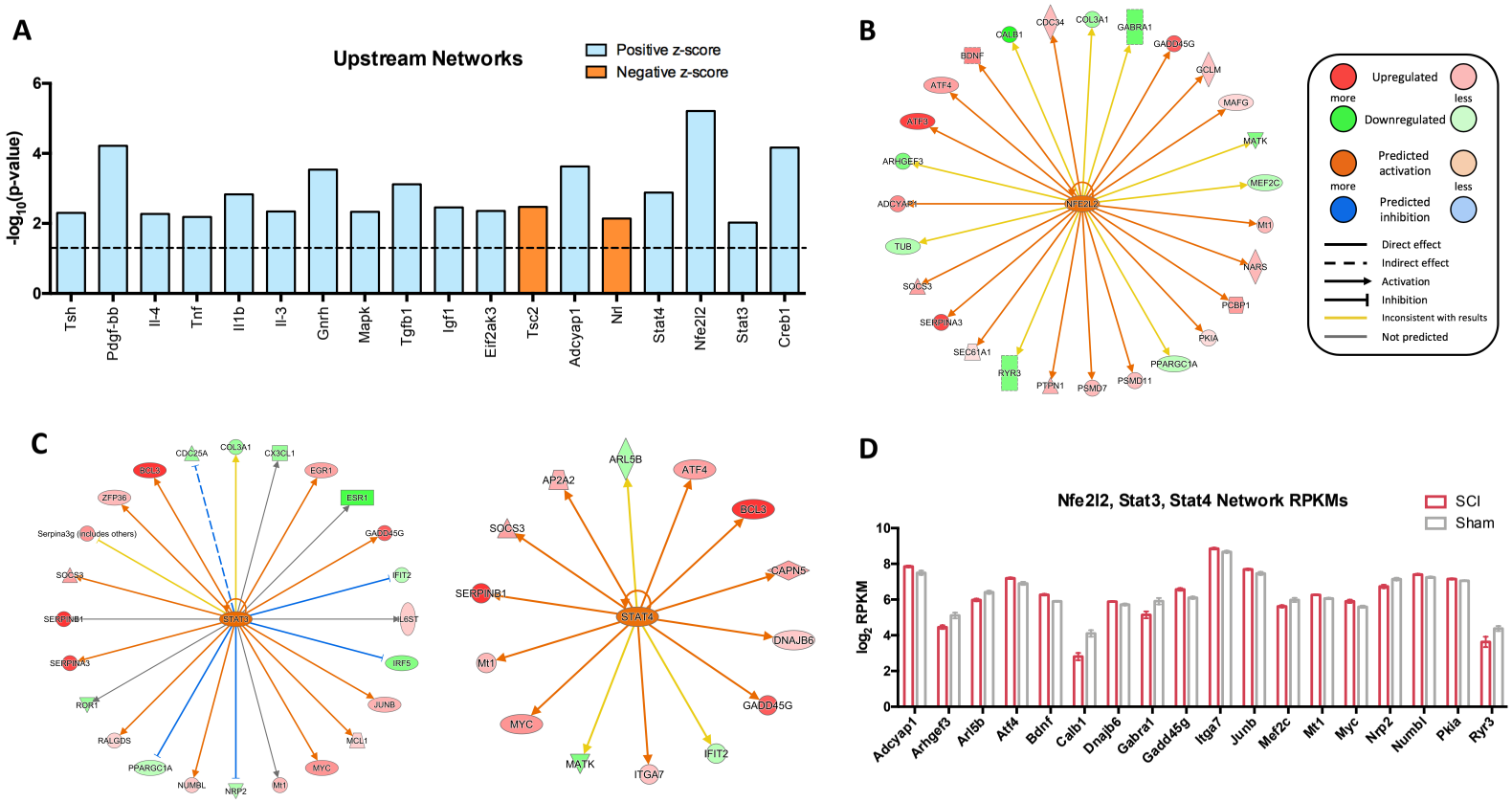

Figure 3-7. Upstream network analysis. (A) IPA network analysis predicted networks based on differentially expressed genes between sham and SCI conditions. Network analysis also made predictions about the activation state of the transcript regulator; positive z-score (blue) indicates activation, negative z-score (orange) indicates inhibition. (B) Transcripts affected by the upstream regulator Nfe2l2 are identified, (C) Stat3 and Stat4 transcript networks. Symbols indicate direct or indirect effects and activation or inhibition of each gene. Color indicates the direction and magnitude of change. (D) Log_2 RPKM values of neuronally relevant genes within the networks shown.

Discussion

SCI may initiate persistent molecular changes in nociceptors, similar to inflammation in models of peripheral injury (Djouhri et al., 2001; Xie et al., 2005). Several studies of SCI pain have evaluated mechanical hypersensitivity from von Frey stimulation above and below the level of thoracic injury, in addition to testing tail withdrawal from heat stimuli (Kramer et al., 2017; Shiao and Lee-Kubli, 2018). Additional behavioral studies have demonstrated that SCI animals exhibit significant increases in mechanical and thermal hypersensitivity compared to naïve and sham animals, beginning at 1 month and persisting for several months post-injury (Bedi et al., 2010; Carlton et al., 2009). However, other studies have asserted that operant behavioral tasks, such as conditioned place preference, are required to effectively study below-level pain in animals (Yezierski, 2005). This study focused on the transition from acute to chronic pain, examining differences that occur at much earlier time points rather than a point at which chronic pain is already present. We first tested behavioral differences between naïve and sham mice to identify changes due to the laminectomy, not the spinal cord injury itself, to better determine a time point that captures the transition from acute to chronic pain following SCI. For example, removal of bone and muscle alone could trigger chronic pain-like symptoms, analogous to post-surgical pain reported in humans (Woolf, 2011). Surprisingly, there were no significant differences between naïve and sham mice at any time point (**Fig. 3-1A,B**), suggesting that the laminectomy did not produce any locomotor differences or behavioral hypersensitivity 1-7 days post-surgery in mice. By contrast, the spinal cord injury produced clear locomotor differences (**Fig. 3-1A2**).

We also considered post-surgical inflammation, using cytokine ELISAs to assess changes in the spinal cord at the level of laminectomy (T8-T11). We wanted to assess changes in cytokine levels for two main reasons; firstly, to confirm that sham mice did not exhibit differences from naïve mice at key timepoints in comparison to SCI mice, and secondly to determine whether sham mice exhibited a prolonged inflammatory response, which could potentially be correlated to the

development of chronic pain (Krames, 2014). Both pro-inflammatory cytokines TNF-α and IL-1β have been studied in neuroprotection models of SCI. IL-6 has been implicated in neurodegeneration after central nervous system (CNS) injury, and the anti-inflammatory cytokine IL-10 exhibits neuroprotective effects (Donnelly and Popovich, 2008; Schmitz and Chew, 2008; Zhang et al., 2019). However, we did not find any significant cytokine changes between naïve and sham mice within 7 days of injury, indicating that the laminectomy did not produce a significant inflammatory response at the time points tested. We also used the cuprizone model as a positive control for the cytokine ELISAs. Cuprizone is a copper chelator that results in the loss of myelin sheath around axons in the CNS (Suzuki and Kikkawa, 1969). Key pathological features of the treatment include secretion of proinflammatory cytokines such as TNF-α and IL-1β (Schmitz and Chew, 2008). Consistent with previous findings, mice treated with cuprizone exhibited significant increases in TNF-α, IL-1β, and IL-10 relative to naïve mice (**Fig. 3-1C**) (Schmitz and Chew, 2008). Tissue injury can also lead to prolonged functional changes and hyperalgesia that are accompanied by behavioral changes due to increased spontaneous activity of nociceptors (Bedi et al., 2010; Carlton et al., 2009; Walters, 2012). Spontaneous activity in nociceptors following SCI begins at 3 days after injury and persists for at least 8 months (Bedi et al., 2010). This increase in nociceptor activity elicits changes within the spinal dorsal horn, which receives input from these nociceptors, ultimately contributing to spontaneous pain (Dubner and Ruda, 1992; Wu et al., 2001). However, the source of hyperexcitability of nociceptors after injury is still unknown. Because spontaneous activity in nociceptors begins at 3 days post-injury, and has been correlated with the generation of persistent pain, we chose to observe transcriptomic changes immediately after this time point at 4 days post-injury (Xie et al., 2005).

Different types of sensory neurons are distinct in their responses to injury. It is likely that, even within an identified subpopulation, cells will nonetheless exhibit heterogeneity (Hu et al., 2016). Despite reported functional and molecular differences, transcriptional profiling of sensory neurons

has been conducted predominantly on whole DRG tissue (Ginsberg et al., 2004; Thakur et al., 2014; Usoskin et al., 2015). Because injury does not impact all afferents in the same way, we analyzed gene expression changes within the population of nociceptors projecting to the skin below the level of injury. Our goal was to better understand how SCI affects molecular changes within a specific population of neurons, and how this may contribute to hypersensitivity following SCI. We focused our analysis on sensory neurons from lumbar DRGs (below the level of the SCI) projecting to the hairy hindpaw skin (**Fig. 3-2A**). After confirming we had isolated the cell population of interest (**Fig. 3-2B, Fig. 3-3**), we used RNAseq to identify changes in gene expression. The use of RNA-Seq has clear advantages over microarrays, since RNA-Seq is not limited to a set of pre-determined transcripts, has a larger dynamic range of transcript expression, and is highly reproducible (Usoskin et al., 2015). By utilizing this technology, we were able to identify transcript changes undetectable with traditional RT-PCR or microarrays (Wang and Zylka, 2009). Our RNAseq data from naïve, sham, and injured animals display distinct patterns of somatosensory genes present in this nociceptor-enriched population. In particular, RPKM values show high levels of Scn10a (a marker for nociceptors), purinergic receptor P2rx3, Mrgprd (markers for the non-peptidergic population of nociceptors), and Calca and Calcb (neuropeptide precursors), indicating that we isolated the desired nociceptor specific cell population, and also indicating important genes within the population of sensory neurons projecting to the hairy hindpaw skin (**Fig. 3-4A-D)**. Multiple gene transcripts important for itch, tactile function, and proprioception all had relatively low RPKM values, indicating again that we isolated the correct cell population, and that injury did not induce modifications in the type of stimuli nociceptors transduce (**Fig. 3-4C**).

Our population level analysis revealed significant changes after SCI in a number of ion channels and receptors that are already known to play a role in pain or hypersensitivity, such as Piezo2, and transcripts involved in excitatory signaling, such as Grik1 (**Fig. 3-5C, Table 3-1**). However,

there were also many genes whose expression and functional roles in persistent pain have yet to be characterized, including Trpc4 and Ttyh1 (**Fig. 3-5A,B, Table 3-1**). Among these comparisons we also observed large fold changes in several genes ((**Fig. 3-6A,B**), highlighted in red and blue). However, instead of focusing on larger changes in a small subset of genes with individual functions, we concentrated our analysis on the interaction of many transcripts that were significantly altered after SCI and how these influenced intracellular signaling pathways **(Fig. 3-6C**).

Ingenuity Pathway Analysis implicated numerous pathways associated with the progression to persistent pain. We took particular interest in the synaptogenesis signaling pathway as a key player at this 4 day time point, suggesting a role for synaptic plasticity in the transition from acute to chronic pain after SCI. In addition to its relevance within our model, many of the transcripts involved in synaptic plasticity overlapped with several other pathways (**Fig. 3-6C**) and had RPKM values that could be validated by qPCR (**Fig. 3-6D**). Synaptogenesis is typically associated with developmental processes, including axon guidance and synapse formation (Klein, 2004). However, activation of various signaling pathways involved in synaptogenesis may also contribute to pain; for example persistent pain is supported via changes in synaptic signaling, neuronal plasticity, and long term potentiation, and may form memory-like networks for painful signals that allow persistent pain to occur long after the initial injury (Khangura et al., 2019; Kobayashi et al., 2007).

Among many of the genes of interest within the synaptogenesis pathway, Ephb2 was significantly down regulated post-injury (**Table 3-1**). The gene transcript is part of the Ephrin tyrosine kinase receptor protein family that is expressed in laminae I-III of the spinal dorsal horn on small and medium sized DRG neurons (presumably nociceptors) (Bundesen et al., 2003). Ephb receptors regulate synaptic activity in the spinal cord and contribute to persistent pain associated with

NMDA activity (Khangura et al., 2019). Numerous receptor tyrosine kinases localize to synapses and contribute to synaptogenesis in addition to EphB receptors, including Trk receptors (Biederer and Stagi, 2008). Ntrk2, the receptor for BDNF, was highly expressed in this cell population; commensurately, BDNF transcript levels were significantly upregulated in the injured population (**Table 3-1**). Camk2g transcript levels were significantly increased in the SCI population of cutaneous nociceptors as well, and recent work has shown phosphorylation of Camk2g induces Bdnf mRNA transcription (Yan et al., 2016). This parallels increasing evidence that neuronal activity (such as increased activity or hyperexcitability) activates alternative neuronal circuits through activity-regulated genes, such as *BDNF* (Lu et al., 2009). Many of the genes significantly altered in the synaptogenesis pathway may function together to generate neuropathic pain (**Suppl. 3-7**).

Regulatory interrelationships predicted by the IPA program were also examined (**Fig. 3-7A-C**). We chose networks implicated in the development of chronic pain and therefore potential gene targets for treatment. Notable is *NFE2L2* (nuclear factor, erythroid derived 2, like 2), because of its association with improper regulation of gene expression and its role in defense against stress in cells (Huppke et al., 2017). Also highlighted are two of the STAT (signal transducer activator of transcription) pathways, *STAT3* and *STAT4*, which are activated in injured nerves by cytokine signaling and associated with neuropathic pain (Dominguez et al., 2008). Activation of *STAT3* in particular has been implicated in the development of chronic pain, recently emerging as a potential target for inflammatory processes in neuropathic pain in the development of therapeutic drugs for pain management (Xue et al., 2014).

Conclusion

Molecular changes typically reflect phenotypic characteristics, and our data show changes in gene expression 4 days after injury, suggesting that many of these genes may be responsible for

the development of spontaneous activity reported elsewhere (Bedi et al., 2010; Yang et al., 2014). We recognize that RNA-Seq of batched neurons elucidated changes in gene targets in a subpopulation of cells, but averaging occurred when pooling large numbers of cells, precluding analysis at the level of the single cell (Haque et al., 2017). Further analysis at the single cell level of cutaneous nociceptors will clarify the contributions of specific subpopulations (non-peptidergic versus peptidergic) to chronic pain after SCI. Functional studies are also needed to analyze the roles of this specific cell population, to better understand the connectivity and plasticity of the CNS and PNS. It is evident that the transition from acute to chronic pain occurs in distinct steps that involve numerous signaling pathways, providing a host of potential new drug targets.

Methods

Animals. Experiments were conducted with adult (8-12 week) female C57BL/6J mice (Jackson Laboratory, Bangor ME). Several chronic pain conditions have a higher prevalence in females, and numerous studies have reported higher pain prevalence in the SCI population among female patients (Cardenas et al., 2004). Women also report greater frequency, severity, and longer lasting pain, as well as neuropathic pain below the level of injury, in comparison to men (Cardenas et al., 2004). The majority of research examines SCI in male rodents and this study will add to what is known in the literature by focusing on female mice (Cardenas et al., 2004). Naïve animals were group housed; sham and spinal cord injured animals were individually caged. All animals were maintained on a 12:12-h light-dark cycle with a temperature-controlled environment, and given food and water ad libitum. All treatments and testing were approved by the University of Connecticut Health Center Institutional Animal Care and Use Committee.

Spinal cord injury (SCI) procedure. Animals were anesthetized by inhalation of isoflurane and a 1.0- cm dorsal midline skin sterile incision was made over T8-T11, as per Ma *et al.* (Ma et al., 2001). Connective and muscle tissue were removed to expose the bone from T9-T10, and a

laminectomy was performed at the T10 vertebral level. Spinal cord injury was produced by compression of the vertical plane of the spinal cord using a vessel clip with a closing pressure of 15g (WPI, Sarasota, FL) for 1.0 minute, exerting pressure from side to side on the spinal cord. Sudden impact is produced by the rapid release of the vessel clip (Tator, 2008). This injury is analogous to the majority of lesions in humans, as the model constitutes both contusion and compression (Marques et al., 2014). After removal of the clip a hemorrhagic ring is present. The wound is closed with coated vicryl absorbable sutures (Ethicon, Somerville, NJ). Mice were allowed to recover in warm cages for 24hr. All animals were administered antibiotics once immediately following surgery (5mg/kg gentamicin), as well as subcutaneous saline for 4 days following surgery, without analgesics. Sham control mice received the same treatment excluding the vessel clip. Manual bladder expression on SCI mice was performed twice daily.

Behavioral tests

Tail-flick test for thermal sensitivity. Mice were acclimated in 50mL tubes for 2 days prior to testing, 20 minutes per day. On testing days, mice were left in their home cage to acclimate to the test room for 30 minutes before testing (Bannon and Malmberg, 2007). Latency to respond to thermal stimuli was measured by dipping the distal 1.5cm of the tail into a 50^{o}C water bath (Ramabadran et al., 1989). The tail was removed from the water upon response, or after 15 seconds to prevent tissue damage. The stimulus was conducted 3 times, at 20 second intervals or less (Zhou et al., 2014). The first response was dropped, and the average latency to respond from two trials was used for analysis. Video recording and VLC software were used to determine tail-flick responses in milliseconds. Mice were tested for thermal sensitivity 1 day prior to surgery for baseline response thresholds, and at days 1, 3, 5, and 7 post-surgery. N=6 per group.

Mechanical sensitivity. To assess mechanical sensitivity, mice were confined in clear plastic containers placed on an elevated wire mesh platform. Prior to testing, mice were acclimated to

the apparatus for 60 minutes. Mechanical reactivity was assessed on the plantar surface of the hind paw using a series of calibrated von Frey filaments according to the up-down method as described (Dixon, 1980), and 50% response thresholds were compared across all conditions. Both hindpaws were tested for mechanical sensitivity, and collapsed across each group of mice per condition. Mice were tested for mechanical hypersensitivity 1 day prior to surgery for baseline response thresholds, and at days 1, 3, 5, and 7 post-surgery. N=6 per group.

Open Field Test. The open field test was conducted using a 16"x16" open-field container subdivided by infrared beams to track movement (San Diego Instruments, San Diego, CA). Data were acquired using the manufacturer's tracking software, which records ambulation movements based on beam breaks as well as central vs. peripheral beam break counts. All mice were placed in the same corner of the box before testing and allowed to freely explore for 10 minutes. Mice were tested 1 day prior to surgery for baseline locomotor behavior, and at days 1, 3, 5, and 7 post-surgery. Spinally injured mice were tested 1 day prior to surgery and 1 day post-surgery. N=3-10 per group.

Cuprizone treated mice. Female C57BL/6 mice (6-10 weeks old) were fed milled chow containing 0.2% bis(cyclohexanone) oxaldihydrazone (cuprizone; Sigma-Aldrich, St. Louis, MO), with food and water available *ad libitum*. Cuprizone feeding was maintained for 35 days to induce demyelination, and tissue was collected for protein analyses on day 35. N=5.

Cytokine ELISAs. Spinal cord segments at the level of laminectomy (T8-T11) were collected from naïve, sham, SCI, or cuprizone treated mice immediately following perfusion with ice cold 0.9% NaCl. Spinal cord segments were homogenized in ice-cold buffer containing 20mM TES, 10mM mannitol, 0.3mg/mL phenylmethylsulfonyl fluoride, 2µg/mL leupeptin, 2µg/mL pepstatin, 2 µg/mL benzamidine, 16µg/mL benzamidine, and 50µg/mL lima bean trypsin inhibitor at a

concentration of 0.1g tissue per 1mL buffer (Mains et al., 2018). Homogenates were freeze-thawed three times, centrifuged (20min, 17,400g), and supernatants were collected. Approximately 60μg of protein per sample was used for each ELISA. The ELISA assays were performed according to the manufacturer's instructions (R&D systems mouse duo-sets IL-10, IL-6, IL-1β, TNF-α, completed with Ancillary Reagent Kit 2 , Minneapolis, MN). The sample absorbance was read with an ELISA plate reader at 450nm; readings were also taken at 570nm to subtract optical background. The concentration was determined based on a standard curve. All results were normalized to amount of protein added per sample and graphed as pg/mg. N= 3-5 per group.

Backlabeling procedure. To backlabel DRG L2-L6 projecting to the hairy hindpaw skin, mice were anesthetized with isoflurane. 0.3% wheat germ agglutinin conjugated to an AF-488 dye (WGA-488, Thermo Fisher, Waltham, MA) in sterile PBS was injected into the sural, common peroneal, and saphenous nerve skin territories for retrograde labeling of DRG neurons (Berta et al., 2017; da Silva Serra et al., 2016). A total of 6μL of WGA-488 was injected 2 days prior to surgery by multiple 2μL injections in the lateral zones of the hindpaw using a 10μL Hamilton Syringe and 30G needle. This technique does not cause significant injury to the sensory afferents being studied.

Primary DRG neuron dissociation. Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) plus xylazine (10 mg/kg) and perfused with ice cold 0.9% NaCl. A laminectomy was performed and L2-L6 DRG from both sides of the spinal column were collected into cold HBSS (KCl 5.4mM, NaCl 137mM, Glucose 5.6mM, Hepes 20mM, pH 7.35 NaOH), after which the mice were sacrificed by decapitation. Sensory neuron dissociation was performed as described (Malin et al., 2007). Briefly, following collection, tissue was treated with 60U papain

(Worthington), 1mg of cysteine, and 6µL of $NaHCO_3$ in 1.5mL HBSS at 37°C for 10 min. Tissue was then treated with 12mg collagenase II (Worthington, Lakewood, NJ) and 14mg dispase (Roche, Basel, Switzerland) in 3mL HBSS at 37°C for 20 min, washed, and triturated with fire polished glass Pasteur pipettes in 1mL of DMEM (Gibco Thermo Fisher Scientific, Waltham, MA) supplemented with FBS (Hyclone, Logan, UT) and pen/strep (Gibco). The cell suspension was pelleted (1 min, 80g), DMEM was removed, and cells were re-suspended in a modified solution (Citri et al., 2011) containing 140mM NaCl, 5mM KCl, 10mM Hepes, 10mM glucose, 0.1% Bovine Serum Albumin, pH 7.4. After re-suspension, cells were strained through a 70µm cell strainer and placed on ice until fluorescence activated cell sorting (FACS) sorting.

Imaging Flow Cytometry. Single cell suspensions of cells isolated from *in situ* WGA-488 labeled DRG were live-stained using Hoechst 33342 (10µg/mL, Thermo Fisher) and propidium iodide (PI) (1µg/mL), and analyzed on an Amnis ImageStreamX Mark II imaging flow cytometer (Luminex Co., Austin, TX). Fluorescent cell images were captured using a 60x objective lens with excitation from a 405nm laser at 20mW power and a 488nm laser at 200mW power. Images of in-focus nucleated WGA AF488-positive cells were identified and electronically gated using IDEAS software (Amnis,v6.2.183, Seattle, WA).

Flow cytometry and cell sorting. Neurons labeled with WGA-488 dye *in situ* in the DRG were purified by fluorescence activated cell sorting. Following primary dissociation of DRGs L2-L6, single cell suspensions were analyzed and sorted using a BD FACS Aria II cell sorter (Becton Dickinson) set up with a 130µm nozzle at 12 PSI in order to gently isolate cells between 10 and 30µm. Single live neurons were defined by electronic gating in FACS DIVA software (BD, ver. 8.01) using forward and side angle light scatter, omission of propidium iodide (PI, 1µg/mL), and AF488 fluorescence. All fluorescence gates were confirmed using fluorescence minus one

controls (e.g.: a sample of cells from unlabeled DRG was used to gate for AF488 positive cells and a sample of cells not treated with PI was used to set the live cell gate). WGA-488 positive cells were sorted directly into lysis buffer (NucleoSpin RNA XS Kit, Machery-Nagel, Bethlehem, PA) and immediately placed on dry ice until RNA extraction.

RNA extraction and RNA sequencing. RNA from FACS sorted cells was isolated using NucleoSpin RNA XS Kit, including a DNA digestion step but without carrier RNA step. Before library preparation RNA quality and integrity was tested for each sample using the Agilent High Sensitivity RNA Screen Tape on the Agilent Tapestation 2200 (Agilent Technologies, Santa Clara, CA). RNA with RIN values ≥6.7 (minimum 6.7, maximum 9.9, average 7.3) was further processed for RNA sequencing. Library preparation was performed using the Illumina sequencing kit for high output 75-cycles for 25-30M total single end reads per sample. DESeq2 analyses (https://bioconductor.org/packages/release/bioc/html/DESeq2.html) of differential expression were performed, and outliers beyond 30-50% of the mean for each group of animals were eliminated (Conesa et al., 2016; Labaj and Kreil, 2016; Love et al., 2014; Wu and Wu, 2016).

Pathway Analysis. Data was analyzed by Ingenuity Pathway Analysis (IPA; Qiagen, Germantown, MD). An overlap of significance for DEseq2 comparisons plus an RPKM cutoff >10 were required for transcripts to be included for IPA analysis. We analyzed 125 transcripts for comparisons between SCI and naïve groups, and 560 transcripts for comparisons between SCI and sham groups.

qPCR validation

Pre-amplification of cDNA for Gene Expression. cDNA was generated from RNA samples from FACS sorted cells with the iScript Reverse Transcription Supermix (#1708840 Bio-Rad, Hercules, CA), N=6 per condition. Target-specific preamplification was performed on cDNA generated from

RNA samples using SsoAdvanced PreAmp Supermix (#1725160 Bio-Rad) containing Sso7d fusion polymerase. Briefly, 20μL of cDNA was preamplified in a total volume of 50μL containing 25μL of 2x SsoAdvanced PreAmp Supermix and 21 primer pairs, 50nM of each primer. Preamplification was performed at 95°C for 3 min followed by 12 cycles of amplification at 95°C for 15 seconds and 58°C for 4 min. Samples were moved directly to ice and stored at -80°C. Preamplified cDNA were diluted 1:5 with H_2O.

qPCR. Following cDNA synthesis and preamplification, qPCR was performed using the primers listed in **Suppl. 3-6**. All primers had calculated melt temperatures of 59.5-63.5C, and all products were 111-143 bp in length, as verified by agarose gel electrophoresis. qPCR was performed at 95°C, 2 min; 95°C, 10 seconds; 55 °C, 15 seconds; and 72°C, 40 seconds, repeating the second through fourth steps for a total of 40 cycles in a Bio-Rad CFX Connect Optics Module machine. iQ SYBR Green Supermix (#1708882 Bio-Rad) was used for linear detection of qPCR results. Hypoxanthine phosphoribosyltransferase (Hprt) was used as the most constant normalizer transcript, based on the RPKM data (Klenke et al., 2016; Lima et al., 2016).

Statistical analyses. Differences between groups were compared using Student's t-test or ANOVA, followed by Tukey's posttest, Bonferroni's multiple comparisons test, or by unpaired Student's t-test. P-values <0.05 were considered statistically significant. Statistics on PCR data were conducted using delta CT values. Heat maps were generated by Microsoft software (Excel) and all other data was plotted using Prism 6 (GraphPad Software, San Diego, CA). R studio was utilized for differential expression analysis; Prism software was used for all other statistical tests.

Acknowledgments

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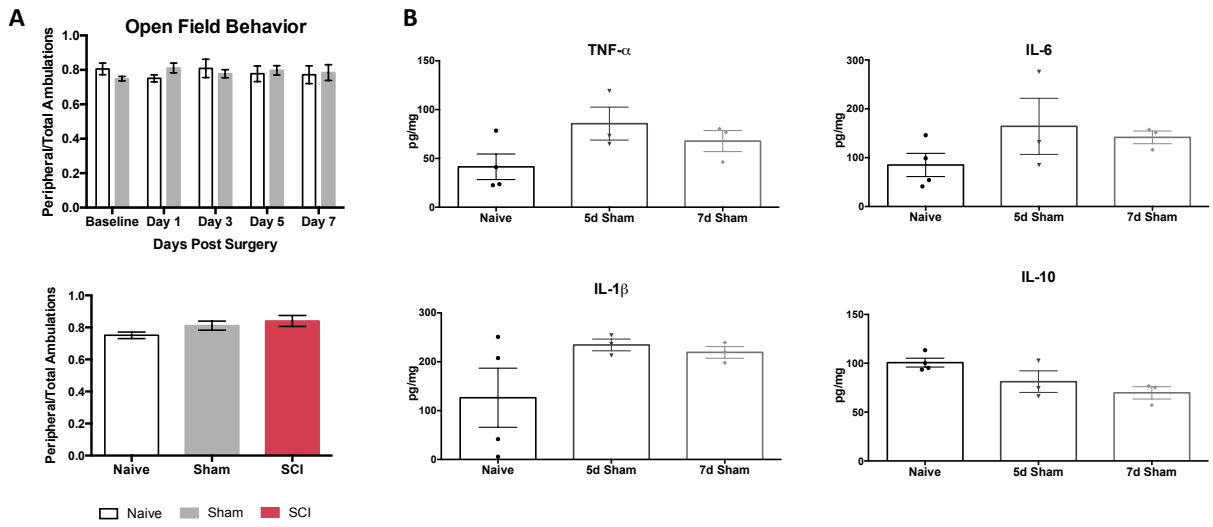
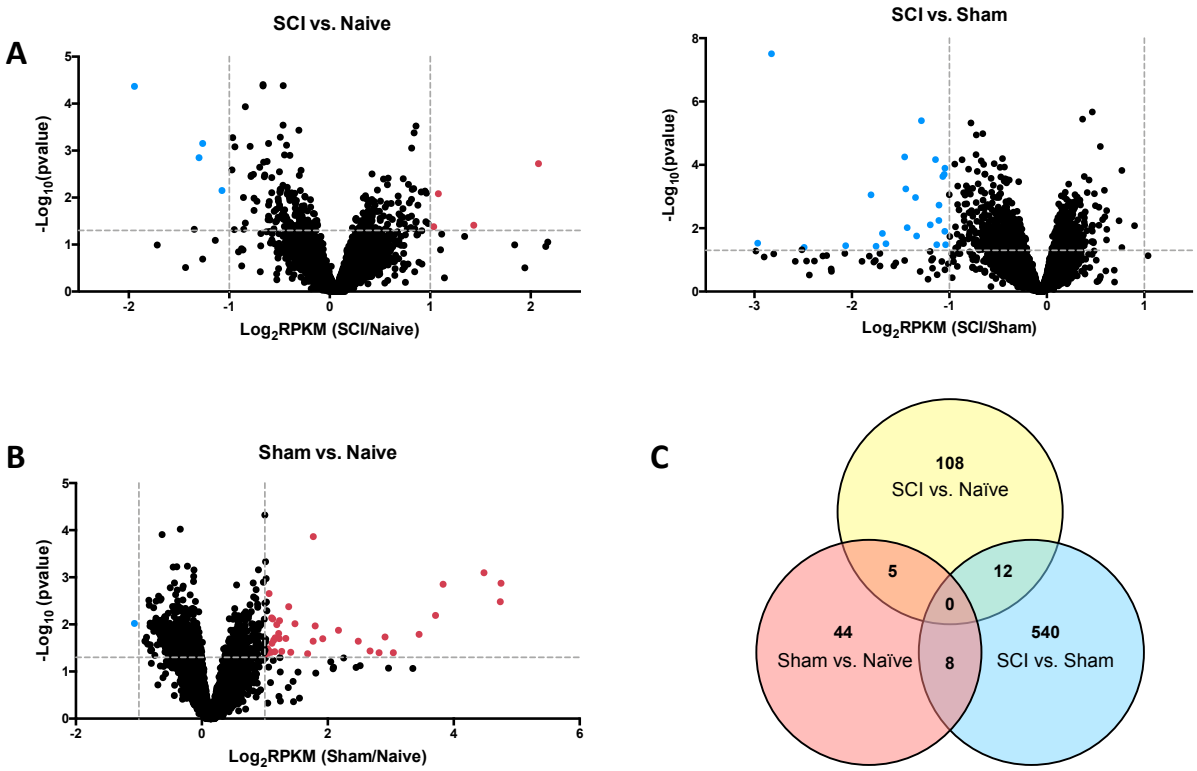
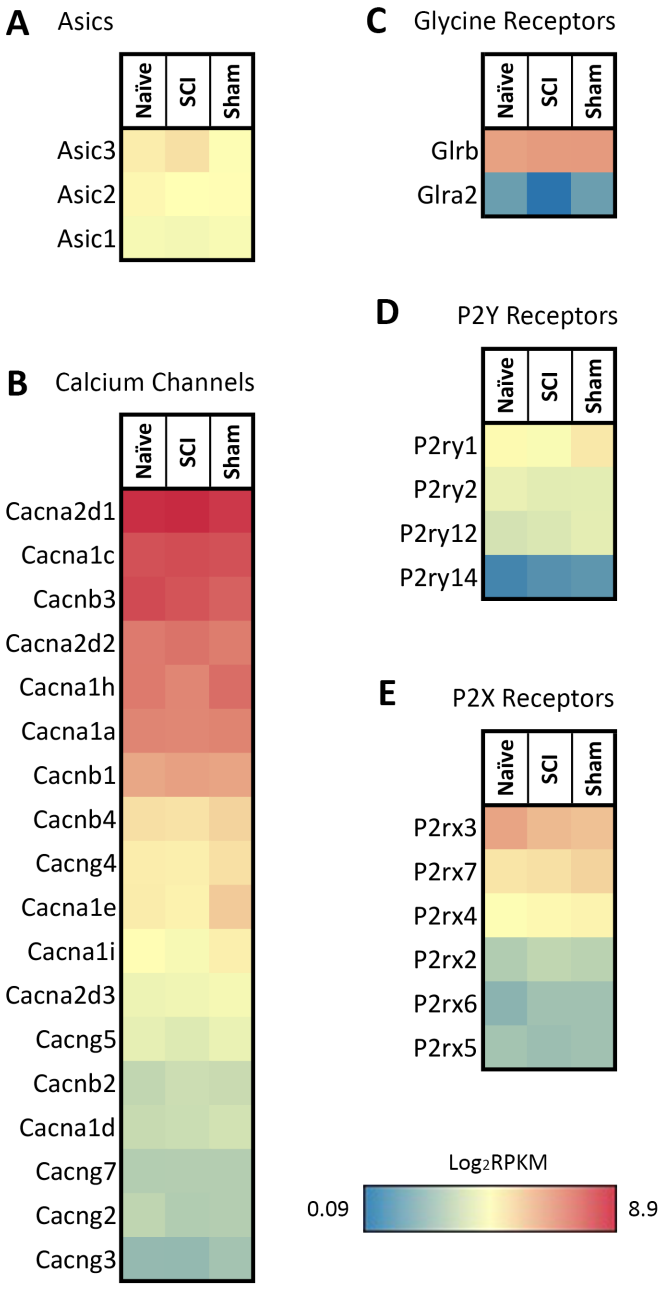
Data Availability: The datasets generated during and/ or analyzed during the current study are available in Gene Expression Omnibus repository under the series record number GSE132552, all other data generated during this study are included in this published article (and its Supplementary Information files).



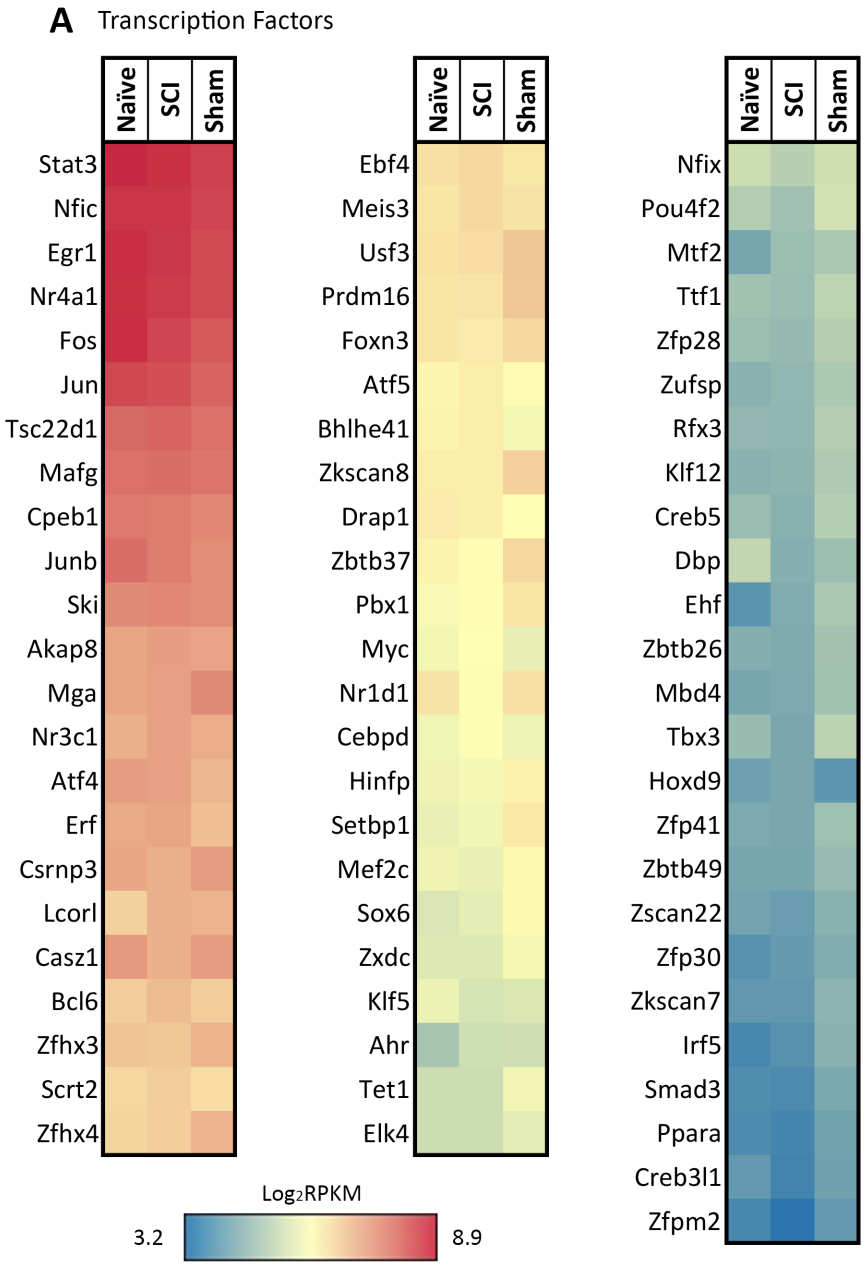
Supplement 3-1. Mobility, cytokine controls. (A) Open field behavior (10-minute trials) conducted on naïve and sham mice 0,1,3,5 and 7 days post-surgery does not differ significantly at any time point in time spent in periphery, N=6 each. Testing done 1 day post-SCI also does not differ significantly in time spent in periphery, N=6 naïve, N=6 sham, N=4 SCI. (B) Cytokine ELISAs on spinal cord segments at the level of laminectomy (T8-T11) show no significant differences between naïve and sham mice 5 or 7 days post-surgery.



Supplement 3-2. Volcano plots, Venn diagram. (A) Volcano plot of RNAseq transcript p-Values calculated by DESeq2 comparing SCI vs. naïve, SCI vs. sham, or (B) sham vs. naïve conditions, RPKM >10. (C) Venn diagram of statistically significant genes from the RNAseq data set determined by an overlap of DESeq2 significant genes ($p<0.05$) and outlier removal, with a cutoff excluding RPKM values <10.



Supplement 3-3. Ion channel heatmaps. Acid sensing ion channels (Asics), calcium channels, glycine receptors, and purinergic receptors (P2Y, P2X). Expression patterns are similar across all three conditions. Despite their known relevance in pain transduction, no significant changes were observed at the 4 day time point tested. RPKM <1 were not included.



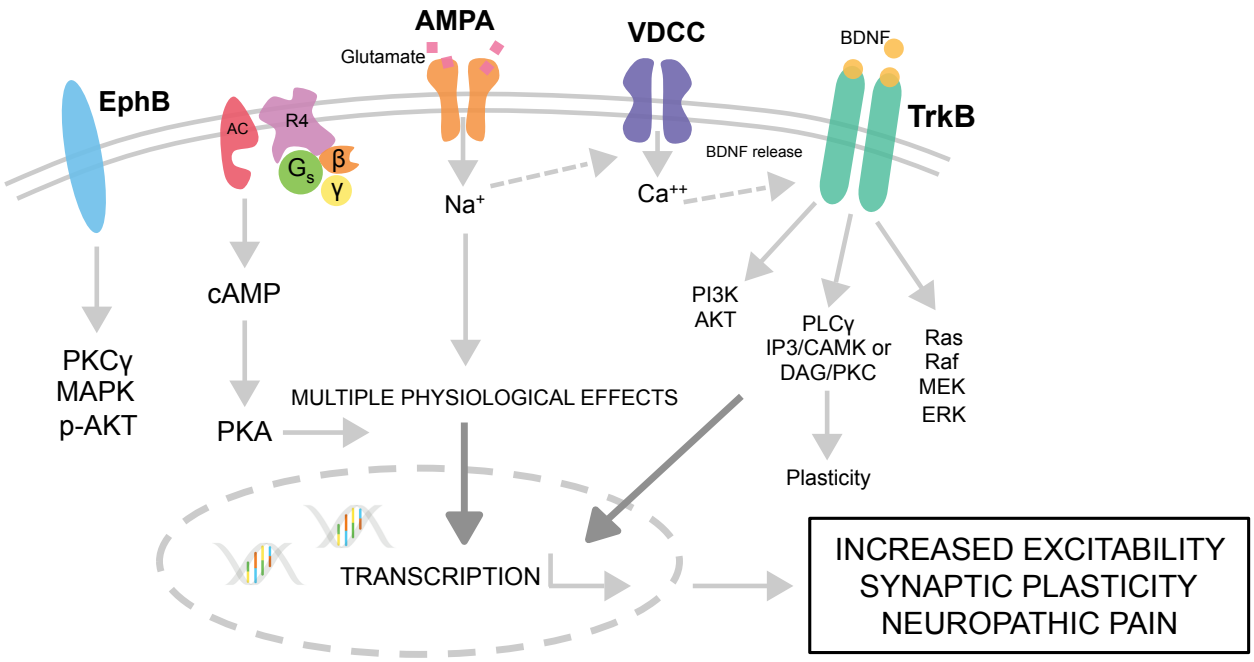
Supplement 3-4. Transcription factor heatmap. Significant changes between SCI vs. naïve or SCI vs. sham conditions by DESeq2: Ahr, Atf4, Cpeb1, Creb3l1, Csrnp3, Drap1, Egr1, Erf, Foxn3, Irf5, Jun, Junb, Mafg, Mef2c, Meis3, Myc, Nr3c1, Nr4a1, Pbx1, Tbx3, Tet1, Zfhx3, Zfp28, Zfp30, Zfp41, Zkscan8, Zscan22. RPKM <1 were not included.

Gene	RPKM Naïve	RPKM SCI	RPKM Sham	p-Value SCI vs. N	p-Value SCI vs. Sham
Ahr	28	39	39	2.0E-02	-
Atf4	154	147	121	-	1.3E-02
Cpeb1	214	208	188	-	1.6E-02
Creb3l1	15	11	17	9.7E-02	1.4E-02
Csrnp3	139	128	155	-	8.4E-03
Drap1	73	71	61	-	4.0E-03
Egr1	448	404	336	-	3.7E-03
Erf	137	141	111	-	1.3E-03
Foxn3	77	73	88	-	3.0E-02
Irf5	12	14	21	-	2.0E-02
Jun	345	323	264	-	7.2E-03
Junb	240	207	178	-	2.5E-02
Mafg	238	242	224	-	4.6E-03
Mef2c	52	49	64	-	1.3E-02
Meis3	77	87	79	-	1.3E-02
Myc	54	60	49	-	1.1E-03
Nr3c1	128	147	132	-	2.0E-02
Nr4a1	435	388	343	-	4.9E-03
Pbx1	56	61	77	-	5.6E-03
Tbx3	25	19	33	1.1E-02	1.7E-03
Tet1	39	39	53	-	3.0E-03
Zfhx3	107	103	124	-	9.7E-03
Zfp28	25	24	32	-	7.3E-03
Zfp30	14	16	20	-	4.0E-02
Zfp41	19	18	26	-	5.5E-03
Zkscan8	71	72	95	-	2.9E-04
Zscan22	17	16	21	-	2.7E-02

Supplement 3-5. Transcription factors. Transcript levels that significantly differ 4 days post-SCI. DESeq2 p-Value based on SCI vs Naïve or SCI vs Sham comparisons. P-Values that are not listed were >0.05.

Genes	Forward	Reverse
Adcy2	AGTCCTCACAGCCTAGGACC	GGCACTAGACACCAAAGTACG
Ap2a2	GCGTGGGGTGGTAAGGTAAT	TCTCTTTTCCACCCAGCCAC
Bdnf	GGCTGACACTTTTGAGCACGTC	CTCCAAAGGCACTTGACTGCTG
Cadm1	GATCCCCACAGGTGATGGAC	GGAGCTGGATCACTGAGTCG
Camk2g	GCACAGGAGCTGGGAAAAGA	GGTCTGAGGCATGCACAAGT
Ephb2	CCGAGTACCAGACCAGCATC	GCTCAAACCCCCGTCTGTTA
Gabrg3	TATCATGCAACGACCCCTGT	TAGATCCTGTGTGTACGGGCA
Gria4	CGCCCAAGGGTTCCTCATTA	CGCTGCCACATTCTCCTTTG
Hprt*	GACTGAAAGACTTGCTCGAGATGTCATG	AGTGCTTTAATGTAATCCAGCAGGTCAGC
Il6st	CTTCGCTCGAGCATGTTTTAGA	AGCCCACGGGCAGAACTA
Kcng3	AGGGAACCCTCCGGGATAAT	TGATGTTCAGGGGTCTCTTGAC
Nlgn2	ATGGCACAAGGCAGGTGAAG	AGGTAAGTTTCCAGGCAGCG
Ntrk2	GCAATCGGGAGCATCTCTCG	GTTGCTGATGACCGAAGCTG
Piezo2	GCACTCTACCTCAGGAAGACTG	CAAAGCTGTGCCACCAGGTTCT
Prkar2b	CCACATGCCCACAAGATATGG	TGAGCTTGCTGGTTGACGTT
Rasgrp1	TCTGCAGGAGGGTATACTGGG	GGTAAAAACCAAGGATGTCAACAGT
Scn10a	ACAGTGATGGTGCTAGGCAA	CAAGTTGTTCACCTCCCCGT
Scn5a	CTAGCTCGAGGCTTCTGCC	GCCGACAAATTGCCTAGCTT
Stxbp2	TCATTAACGCTGAGAACCTGGG	ACCTCTCTATCTCCTCTGCCAA
Syt4	CTCATCGCCATCCAGTGACA	TGTACATACATGCAGAGGCCG
Trpc3	AAAGCGTCACTGAGTCGTGT	GAGGCCGGAAAGGTTCTCAT

Supplement 3-6. Primer list. Primers for voltage-gated channels, receptors, Trp channels, or involvement in the synaptogenesis pathway were designed for PCR products 111-143 bp in size, Tm=59.5-63.5C, validated on whole DRG tissue before preamplification. Hprt was the least variable gene based on RNAseq results.



Supplement 3-7. Proposed model of how this signaling pathway may be contributing to the onset of chronic pain 4 days post-SCI in DRG distal to the site of injury. RNAseq data, IPA analysis, and qPCR validation suggest Ntrk2 (TrkB) signaling may play a role during the transition from acute to chronic pain at 4 days post-SCI.

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CHAPTER 4

Dissecting the Roles of Kalirin-7/PSD95/GluN2B Interactions in Different Forms of Synaptic Plasticity

This chapter was submitted to *Journal of Neuroscience*. Electrophysiological work was performed by Dr. Mason Yeh. Biochemical work was initiated by MLY; pursued, expanded and completed by JRY, with advice from BAE and REM. Initial draft was written by MLY and REM; edited and approved by MLY, JRY, ESL, BAE, and REM.

Abstract

Kal7 is a Rac1/RhoG GEF localized to the postsynaptic density and plays an important role in synaptic plasticity. Behavioral phenotypes observed in Kal7 knockout mice are quite specific, and following genetic ablation of Kal7, there is a decrease in spine density with corresponding impairments in structural and functional synaptic plasticity. Further works has associated loss or improper function of Kal7 to numerous diseases, including schizophrenia, autism, stroke, and intellectual impairments. It is evident by these studies that Kal7 plays a fundamental role in synaptic structure and function in addition to its roles in neuronal stability and growth. However, the underlying mechanisms are still not fully understood. In order to address this question, we administered intracellular blocking peptides to mimic acute modifications of the presence of Kal7 at the synapse, to observe if plasticity deficits that have been reported in Kal7 knockout mice are the product of a long series of developmental processes, can be disrupted by brief interference by peptides mimicking interacting sequences, or some blend of possibilities. We found that specific, brief (10 minutes) disruption of Kal7 with PSD-95 or GluN2B results in significant electrophysiological changes in long-term potentiation and long-term depression. Biochemical studies indicate that Kal7 interacts with PSD-95 at multiple sites within Kal7, despite the changes in specific interactions between binding sites that we observed at the electrophysiological level.

Introduction

Several genetic studies have suggested a role for Kalirin in a variety of neurodegenerative and neuropsychiatric diseases including schizophrenia, autism, stroke, and intellectual impairments (Cahill et al., 2012). Kalirin is a large gene with numerous alternate splicing forms. Kalirin-7 (Kal7) is the most highly expressed Kalirin isoform in the adult mammalian brain, exhibits highest expression levels in the cerebral cortex and hippocampus, and is required for cortical pyramidal neuron spine morphogenesis (Miller et al., 2015; Miller et al., 2013; Miller et al., 2017b). Electrophysiological studies have demonstrated that Kal7 is necessary for both long-term potentiation (LTP) and long-term depression (LTD), both of which require glutamate receptors containing a GluN2B subunit (Kiraly et al., 2011; Lemtiri-Chlieh et al., 2011). This suggests a major role for Kal7 in synaptic plasticity, learning, and memory.

Background

Excitatory synapses are characterized by the post-synaptic density (PSD), which are typically located at the tips of dendritic spines. PSD-95 is a member of the membrane-associated guanylate kinase protein family (MAGUKs). MAGUKs play a role in the organization of multi-molecular signaling complexes via multiple protein-protein interaction domains. Different PDZ domains of PSD-95 are responsible for discrete functions. Each protein is comprised of three PDZ domains at the NH_2-terminal, one SH3 domain in the middle, and an enzymatically inactive COOH-terminal guanylate kinase domain at the COOH-terminal (Kornau et al., 1997). Several proteins that bind PDZ domains are able to do so directly via the last several residues at the C-terminal end. This includes the NMDA receptor (NMDAR) subunits GluN2A and GluN2B, which bind to a PDZ domain of PSD-95 by interacting with the carboxyl-terminal tails of the GluN2 subunits. (**Fig. 4-1**) (Irie et al., 1997; Songyang et al., 1997).

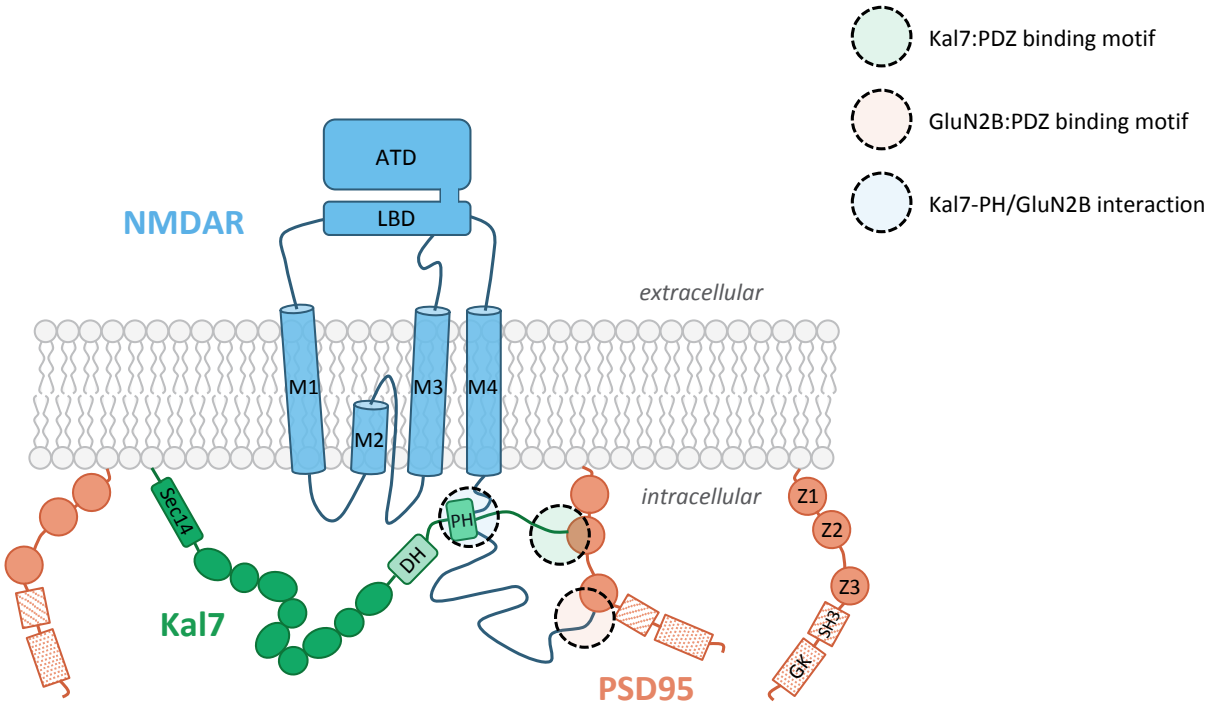

Figure 4-1. Schematic depicting the interactions between NMDAR, Kal7, and PSD-95. The NMDAR contains GluN2B subunit that terminates with PDZ binding motifs at its C-terminal end. Kal7 also intersects with PDZ binding motifs that interact with PSD-95 within the intracellular space. While both share an interaction with the PDZ domain of PSD-95, GluN2B and Kal7 interact directly at the PH1 region of Kal7.

NMDARs are glutamate receptors located in the PSD. They are permeable to calcium and play a key role in synaptic plasticity. It is well established that activation of NMDAR triggers a signaling cascade that recruits AMPA receptors (AMPAR) into the PSD and results in LTP (Sheng and Hoogenraad, 2007). Likewise, weaker prolonged activation of NMDAR is known to prompt reduction of AMPARs in the PSD, and results in LTD (Sheng and Hoogenraad, 2007). PSD-95 may facilitate NMDA receptor clustering at synapses via this interaction as well as facilitating NMDA coupling with downstream signaling molecules, including Kal7 (Irie et al., 1997; Sheng and Kim, 2011).

Previous work has demonstrated that PSD-95 and Kal7 also interact (**Fig. 4-1**) (Penzes et al., 2001). Four major isoforms of Kalirin are expressed in the adult central nervous system (CNS) (Penzes et al., 2000). All four isoforms contain a GEF domain that activates Rac1 (Rabiner et al., 2005). The activation of Rac1 allows the molecule to bind to p21-activated kinase (PAK), which, once activated, cooperates in actin remodeling. Differences in localization of the Kalirin isoforms, as well as differences in the functional domains in each isoform, indicate distinct regulatory roles for development and synaptic plasticity. Elevated levels of Kal7 are found in dendritic spines, and its expression increases during synaptogenesis, while Kal9 expression is primarily located throughout dendritic processes and the cell body, and Kal12 is predominantly isolated to the cell soma (Miller et al., 2013; Penzes et al., 2000).

Recent work has established Kal7 as a primary constituent of proper dendritic spine development and structural and functional plasticity (Miller et al., 2017b). The protein is known to play a fundamental role in neuronal stability and growth, including maintenance of neuronal dendrites and dendritic spines in the hippocampus (Russell et al., 2014; Youn et al., 2007). Kal7 interacts directly with numerous synaptic proteins, including PSD-95 via a unique PDZ-binding motif, which interacts with the PDZ domains in PSD-95. (Miller et al., 2017a). Experiments done by Kiraly et

al. demonstrated the interaction between the pleckstrin homology domain of Kal7 (Kal7-PH) and the juxtamembrane region of GluN2B (the membrane-proximal portion of the COOH-terminal intracellular tail) (Kiraly et al., 2011). This was accomplished by a variety of co-immunoprecipitation (co-IP) experiments using transfected HEK293 cells with constructs expressing Kal7, Kal7-PH domain, GluN2B, and GluN2A. The co-IPs demonstrated that Kal7 co-precipitates with GluN2B but not GluN2A, and that the Kal7-PH domain alone is capable of co-precipitating with GluN2B, suggesting binding specificity. Additional co-IPs confirmed that Kal7-PH binds to a construct expressing only the membrane-proximal portion of the COOH-terminal tail of GluN2B, but not to constructs expressing other regions of GluN2B (**Fig. 4-1**), validating the interaction between Kal7-PH and the juxtamembrane region of the COOH-terminal tail of GluN2B specifically. It is evident by these interactions that Kal7 plays a fundamental role in synaptic structure and function in addition to its roles in neuronal stability and growth. This has been demonstrated in several studies where, following genetic ablation of Kal7, there is a decrease in spine density that is accompanied by impaired structural and functional synaptic plasticity (Lemtiri-Chlieh et al., 2011; Lu et al., 2015; Ma et al., 2008).

Aim of study

Alterations in the shape, size, and function of dendritic spines elicit changes in synaptic transmission and plasticity, as well as learning and memory (Sheng and Hoogenraad, 2007). Improper development of dendritic spine morphology and plasticity also contributes to a variety of neurodevelopmental and neuropsychiatric disorders. Because of this, the underlying mechanisms regulating spine plasticity has been of great interest. Kal7 may adhere membrane proteins to the actin cytoskeleton and provide a connection between postsynaptic membrane signaling and cytoskeletal rearrangements that occur during synaptic plasticity (Penzes et al., 2000).

LTP and LTD are extensively studied forms of synaptic plasticity, providing a potential means for understanding the cellular basis for learning and memory, but the underlying molecular mechanisms are still not fully understood (Zhou et al., 2018). Electrophysiological studies on CA1 hippocampal neurons lacking Kal7 have documented suppressed LTP in response to theta burst stimulation, but still demonstrate normal neuronal electrical membrane properties (Lemtiri-Chlieh et al., 2011). Additional studies have shown that LTD is also diminished in Kal7$^{-/-}$ mice, as well as replicating this phenomenon outside of the hippocampus in the spinal cord (Lemtiri-Chlieh et al., 2011; Lu et al., 2015). Mice lacking Kal7 lack nociceptor-dependent LTP in the dorsal horn of the spinal cord, and wildtype mice injected in the spinal cord with siRNA targeting Kalirin exhibited decreased pain-like phenotypes (Lu et al., 2015). Further work used intracellular infusion of a Kal7-specific interfering peptide to block spinal pain LTP, which is the approach we have used in these electrophysiological studies using hippocampal slices (**Fig. 4-2**) (Lu et al., 2015).

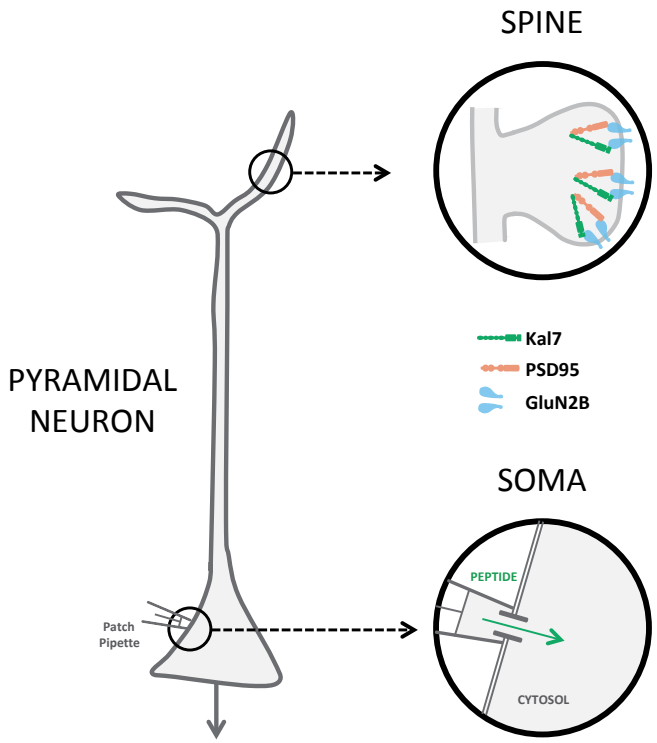

Figure 4-2. Depiction of physiological approach taken to determine whether acute application of intracellular interfering peptides has an effect on LTP or LTD. Peptides were diluted into intracellular recording solution and injected into hippocampal pyramidal neurons.

While previous studies have shown that disturbing the expression of Kal7 disrupts synaptogenesis, we wanted to examine if acute disruption of specific interactions between Kal7, NMDA, and PSD-95 with synthetic peptides could mimic the deficits observed in hippocampal synaptic plasticity in Kal7$^{-/-}$ mice, or if the plasticity deficits are dependent on the prolonged absence of Kal7 beginning during synaptogenesis. Disruption of protein-protein interactions is a practical approach to manipulate specific cellular functions while circumventing adverse effects with the use of non-specific drugs. Thus, we utilized synthetic peptides designed to mimic the C-terminal PDZ-binding motifs of Kal7 and GluN2B and the PH-domain of Kal7, which interacts with the GluN2B subunit (Kal7-GluN2B), to interrupt the Kal7 and PDZ binding motif, the GluN2B and PDZ binding motif, or the Kal7-PH domain and GluN2B interaction site (**Fig. 4-2**) (Houslay, 2009).

Summary of electrophysiological results

Electrophysiological whole cell patch clamp experiments in the CA1 of mouse hippocampal slices by Dr. Mason Yeh confirmed our hypothesis that acute disruption of Kal7 and PSD-95 interactions with an interfering peptide is capable of blocking synaptic plasticity. Peptides designed to interfere at other interaction sites of GluN2B and PSD-95 as well as Kal7 and GluN2B interactions are also capable of blocking LTP (summarized schematically in **Fig. 4-3**). Dr. Yeh's additional experiments strongly suggested that interactions between PSD-95, GluN2B and Kal7 differentially mediate LTD. These experiments indicated that interference with unfettered Kal7 binding to one or more unidentified partner is required for expression of both LTP and LTD in the CA1 of mouse hippocampus. In addition, the interference by small mimicking peptides was dose dependent (low concentrations of blocking peptide did not elicit significant changes in LTP) as well as induction paradigm dependent (Kal7-mimicking and GluN2B-mimicking peptides did not elicit changes with high frequency stimulation, but did elicit changes using theta burst stimulation).

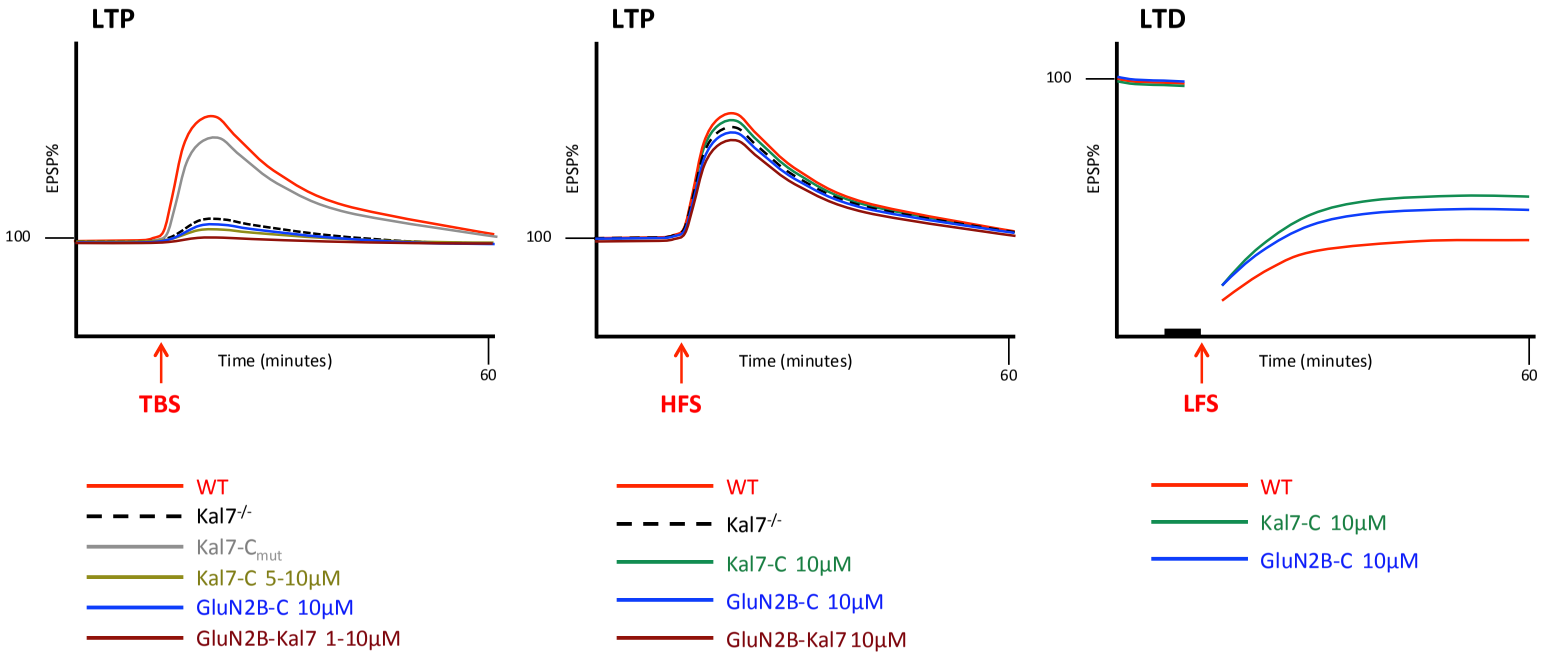

Figure 3-3. Electrophysiological recordings revealed significantly impaired LTP in Kal7$^{-/-}$ mouse and interfering peptides designed to mimic interruptions at key binding motifs including the C-terminal PDZ-binding motif of Kal7 (Kal7-C) or GluN2B (GluN2B-C) , a mutated Kal7-C$_{mut}$, and the PH-domain of Kal7 which interacts with GluN2B (Kal7-GluN2B) using a theta-burst stimulation (TBS) paradigm to elicit LTP. High frequency stimulation (HFS) stimulation did not elicit any significant changes between WT mice, Kal7$^{-/-}$ mice or WT mice with interfering peptides in the recording pipettes. Low frequency stimulation (LFS) produced LTD in control neurons, while injection of interfering peptide abolished LTD. It is important to note that all responses produced by intracellular injection of peptide were also concentration dependent.

Potential biochemical basis of the electrophysiological results

We next wanted to address the biochemical interaction of Kal7 and GluN2B, more specifically if GluN2B and Kal7 compete for binding sites on PSD-95, and if this could explain the electrophysiological results that utilized the same interfering peptides. Because Kal7 and GluN2B both have PDZ binding motifs that interact with PSD-95, the first approach to address the biochemical basis of these interactions was to disturb PDZ binding to Kal7 with interfering peptides. Binding studies were designed several ways, using both bacterially-expressed and mammalian cell-expressed proteins, along with synthetic peptides. Initially, we bound biotinylated GluN2B peptide to NeutrAvidin-Agarose resin and tested binding of a protein encoding PDZ1 (the first PDZ domain of PSD-95), PDZ2, PDZ3 or the full PDZ123 (the 3 PDZ domains of PSD-95 without the SH3 and MAGUK domains) expressed in BL21/DE3 bacteria with different concentrations of interfering GluN2B-related peptides (Walkup et al., 2016). We also tried the mammalian version, binding PSD-95 protein (from transfected HEK293 cells) to GluN2B-peptide-containing resin or ELISA plates, paired with different concentrations of candidate soluble GluN2B interfering peptides. We immobilized bacterial PDZ123 or mammalian PSD-95 to NeutrAvidin-Agarose, SpeedBeed magnetic NeutrAvidin, and UltraLink Biosupport resins to monitor binding of purified Kal7 (from baculovirus expression (Miller et al., 2017a)) paired with competing GluN2B peptides and control peptides (several biotinylated peptides from within the Kal7 sequence). However, we could not demonstrate any competitive binding above controls for any combination of immobilization technique, ligands and binding targets.

These results suggested that the original hypothesis was incorrect and perhaps PSD-95 was interacting with Kal7 and GluN2B through additional sites other than the COOH-terminal PDZ binding domains, or that multiple PDZ domains were in play and that interfering with a single domain was inadequate to disrupt the overall interaction. We took advantage of the fact that Kal8 lacks the C-terminus PDZ binding domain of Kal7; we utilized expression vectors for Kal7, Kal8

(Miller et al., 2015; Miller et al., 2017a; Miller et al., 2017b), or PSD-95 (FLAG-tagged; Addgene, #15463), separately transfected these into HEK293 cells, and made protein extracts. The premise was that immobilizing peptides and proteins produced structural changes incompatible with native binding. We then used co-immunoprecipitation to pull down gently solubilized PSD-95 in combination with either gently solubilized Kal7 or Kal8 proteins (**Fig. 4-4**). The gels demonstrated that both Kal7 and Kal8 proteins interacted specifically with PSD-95, regardless of whether or not they have a PDZ binding motif.

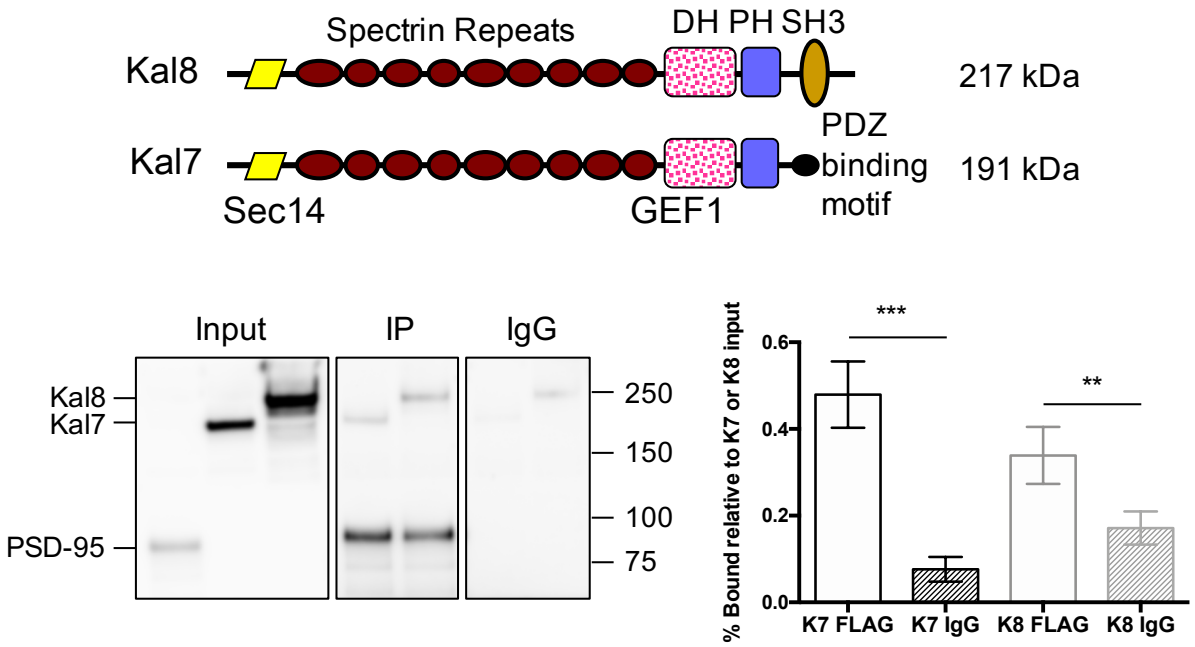

Figure 4-4. PSD-95 associates with both Kal7 and Kal8 *in vitro*. Immunoprecipitation (IP) was performed on FLAG-tagged PSD-95, Kal7 (K7), and Kal8 (K8) protein extracts from transfected HEK293 cells using FLAG antibody or a comparable amount of nonimmune rabbit IgG. Co-IP revealed interactions between PSD-95 with both Kal7 and Kal8, regardless of the presence of a COOH-terminal PDZ-binding domain; IgG controls show no significant binding of protein. Co-IP FLAG antibody of Kal7 or Kal8 with PSD-95 is significantly greater than control IgG antibody; students paired t-test, p=0.0008 [***] and p= 0.0031 [**], respectively. Unpaired t-test comparing bound Kal7 FLAG with bound K8 FLAG was not statistically significant, p= 0.1890. N=7.

Our data illustrate that appropriate protein domain interactions are crucial for proper signal complex formation and localization to confer correct function. While specific interactions between binding sites may be suggested at the electrophysiological level of a single cell, larger scale biochemical experiments elucidate that these molecules have more complex binding capabilities. The notion that small molecules are able to disrupt specific interactions of PDZ domains may still present potential therapeutic applications without triggering greater changes at the PSD (Houslay, 2009).

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CHAPTER 5

Future Directions

The data presented in chapters 1-3 have a common theme that focuses on capturing molecular changes during the transition from acute to chronic pain following spinal cord injury (SCI). Chapter 3 in particular emphasizes these underlying molecular changes in an unbiased manner in the nociceptor population of cells that projects to the hairy hindpaw skin by utilizing RNA sequencing. However, there are still many aspects of identifying this cell population that this work did not address.

A natural next step in further defining this cell population is to study the population at the single cell level using RNAseq or Fluidigm technologies. Sensory neurons display a multiplicity of responses after injuries, which are discrete to specific subsets within populations. By again utilizing FACS sorting, but sorting instead for individual neurons from dorsal root ganglion (DRG), we could observe transcriptional changes at the single cell level. This could be accomplished two ways, either through RNAseq or by using Fluidigm technologies with a customized panel of gene targets determined from the RNA-Seq results generated in chapter 3. Both approaches would better help define the subpopulations, as well as their individual contributions to different aspects of pain transduction (for example heat or mechanical). This is in addition to being able to define each cell as belonging to peptidergic or non-peptidergic nociceptor populations.

The existing data also do not address if there are changes in physiological response profiles of individual cutaneous nociceptors following spinal cord injury. Previous work has demonstrated an increased prevalence of spontaneous activity (SA) in cell bodies of sensory neurons following SCI, and has established that persistent activity from afferent fibers contributes to the

development and maintenance of chronic pain following SCI. Future work could be aimed at defining the physiological responses of these backlabeled cells. Demonstrating that this cell specific population exhibits SA would correspond with previous literature, as well as establish a physiological profile that matches the transcriptional changes that were observed.

Persistent activity of nociceptors, as well as transcriptional changes, can be observed by the manifestation of mechanical and thermal behavioral hypersensitivity. Additional work could investigate the manifestation of chronic pain behaviors at later time points, several weeks or months post-injury. To utilize the data presented, potential gene targets of interest, such as Ntrk2, could be blocked by inhibitors or knocked down by small interfering RNA (siRNA) to test if modifying genes that change early on will help prevent the onset of pain at later time points.

Similarly, additional time points during the transition from acute to chronic pain, such as 3 ,5, or 7 days post-injury, could be studied in the same way as the data presented to examine if transcript changes are similar to those found in the data presented. It is possible that different pathways and genetic markers are present at different time points that are equal contributors to the onset of chronic pain.

Another important aspect to consider is that the data presented examine changes in only female mice. While there is a higher prevalence of reported pain in the female patients, the risk for SCI is higher in males. Studying both male and female populations will provide data on possible common genetic markers for potential treatment of chronic pain, but will also distinguish sex differences that may also contribute to chronic pain after SCI. This approach could also perhaps offer an explanation for why chronic pain is more prevalent in females.

It is evident that SCI alters genetic, cellular, and molecular pathways, all of which contribute to the development of chronic pain. Because of this, a multifaceted approach is needed to better understand the underlying mechanisms of SCI pain and to offer improved treatment options.